CRANFIELD UNIVERSITY

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Investigations of the inflammatory pathogenesis of age related macular degeneration and a therapeutic role for Minocycline

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ABSTRACT

Age related macular degeneration (AMD) is the leading cause of blindness in people over the age of 50 in the western world. The wet form of AMD is associated with choroidal neovascularisation. The pathogenesis of choroidal neovascularisation is complex involving neovascular growth, vascular leakage, hypoxia and inflammation. Evidence suggests that immunological events play a key role in the pathogenesis of AMD. In AMD a chronic low grade inflammatory process may instigate the pathophysiological process culminating in eventual visual loss. Minocycline is a tetracycline derivative with anti-inflammatory in addition to antibiotic effects. This study investigated the effects of minocycline on retinal pigment epithelial cells in culture. Cell viability and apoptosis was studied with flow cytometry. Cells were exposed to glycated albumin and hypoxia as these processes occur during ageing. The effects of minocycline on IL-8 and MCP-1 production from ARPE-19 cells in culture were investigated with enzyme linked immunosorbent assay.

The results showed a potential narrow therapeutic window for minocycline to act on retinal pigment epithelial cells. Cell viability decreased rapidly at minocycline doses above 5µM. Minocycline suppressed the production of inflammatory cytokines IL-8 and MCP-1 in cell culture.

A clinical trial was conducted to investigate whether combination therapy aimed at targeting different pathways in the AMD disease process would be effective. This trial was powered to determine adverse events when a quadruple therapy

of reduced fluence photodynamic therapy (PDT), intravitreal ranibizumab, dexamethasone and oral minocycline were used as treatments.

The clinical trial demonstrated that anti-VEGF treatment administered in combination with other agents was not as effective as monthly anti-VEGF monotherapy at sustaining visual improvement. However, the trial did demonstrate that combination therapy could be delivered safely.

The results demonstrate that minocycline has a potential therapeutic role for the inflammatory changes in neovascular age related macular degeneration.

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List of Abbrevations

%	percentage
°C	degrees centigrade
µg	microgrammes
µM	micromolar
661W	immortalized mouse photoreceptor cell line
ABCR	ABCR gene
ADM	Adrenomedullin
AE490	selective inhibitor of jak2
AGE	Advanced glycation end products
AIF	Apoptosis inducing factor
Akt	protein kinase B
AMD	Age related macular degeneration
ANCHOR	Anti-VEGF antibody for the treatment of predominantly
ANOVA	Analysis of variation
ApoE	Apolipoprotein E
AREDS	Age Related Eye Disease Study
ARPE-19	Human retinal pigment epithelial cell line
ATF-2	Activating transcription factor 2
ATP	adenosine triphosphate
ATPγS	Adenosine 5'-O-(3-thio)triphosphate
ATTC	American type tissue collection
BAY 11-7085	inhibitor of nuclear factor kappa beta
Bcl-2	B cell lymphoma-2 (anti-apoptotic protein)
BCVA	best corrected visual acuity
	bromide assay
C	concentration
$CaCl_2$	Calcium chloride
CAD	Caspase activated deoxyribonuclease
cAMP	cyclic adenosine monophosphate
CAPE	caffeic acid phenethyl ester
CATT	Comparion of age related macular degeneration treatment
CABERNET	A multicentre randomised controlled trial evaluating the safety and efficacy of epimacular brachytherapy in treatment naive patients
CCL2	C-C motif ligand 2
CCR2	C-C motif receptor 2
CD3	Cluster of differentiation
CFH	Complement factor H
c-Fos	member of the Fos family of transcription factors
c-Jun	protein, encoded by the JUN gene family, component of transcription factor complex
	classic choroidal neovascularisation in AMD
CMT	Chemically modified non-antibiotic tetracyclines
CNS	Central nervous system

CNV	Choroidal neovascularisation
CO_2	Carbon dioxide
COX-2	cyclooxygenase-2
c-REL	protein member of the nuclear factor kappa beta family of transcription factors
CRP	C reactive protein
CRT	central retinal thickness
CTGF	Connective tissue growth factor
CV	Coefficient of variation
CX3CL1	C-X3-C motif ligand 1
CX3CR1	C-X3-C motif receptor 1
DENALI	A randomised multicentre North American trial in patients with subfoveal choroidal neovascularisation secondary to age related macular degeneration, to evaluate whether verteporfin photodynamic therapy combined with ranibizumab was not inferior to monthly ranibizumab monotherapy
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMB	Epimacular brachytherapy
ERK	Extracellular regulated kinase
EPO	Erythropoietin
ETDRS	Early treatment of diabetic retinopathy study
EVEREST	Efficacy and safety of verteporfin photodynamic therapy in combination with ranibizumab or alone versus ranibizumab monotherapy in patients with symptomatic macular polypoidal choroidal vasculopathy
FA	Fluroscein angiography
Fab	Fragment antigen binding
Fas/CD95	Fas cell surface death receptor/ cluster of differentiation 95
FAZ	Foveal Avascular Zone
FBS	Foetal bovine serum
Fc	Fragment crystallisable
FDA	Food and Drug Administration
FGF2	Fibroblast growth factor 2
FITC	Fluroscein isothiocynanate
FOCUS	Study designed to evaluate the safety, tolerability, and efficacy of ranibizumab treatment in conjunction with verteporfin photodynamic therapy compared with verteporfin photodynamic therapy alone in patients with subfoveal, predominantly classic choroidal neovascularisation secondary to age related macular degeneration

FRA-1	member of the Fos family of transcription factors
FRA-2	member of the Fos family of transcription factors
g	grammes
GHSA	glycated human serum albumin
G-protein	bind nucleotides guanosine-5'-triphosphate (GTP) and guanosine-5'-biphosphate (GDP)
H_2O_2	Hydrogen peroxide
HbA1c	Glycosylated haemoglobin
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor
HIF-1α	Hypoxia inducible factor 1 alpha
HLA-DR	Major histocompatibility complex cell surface receptor encoded by the human leukocyte antigen complex
HOG	high osmolarity glycerol
HREs	Hypoxia response elements
HRPC	human retinal progenitor cells
hRPE	human retinal pigment epithelial
HRPE	Human retinal pigment epithelium
Hsp 27	Heat shock protein 27
Hsp-70	Heat shock protein 70
HUVEC	human umbilical vein endothelial cells
IAPS	Inhibitor of apoptosis proteins
ICAD	Inhibitor of caspase activated deoxyribonuclease
IF-γ	Interferon gamma
IGFs	Insulin like growth factors
IgG	Immunoglogulin G
IκBα	inhibitory subunit of NFκB
IκBβ	inhibitory subunit of NFκB
IL-10	Interleukin 10
IL-18	Interleukin 18
IL-1β	Interleukin 1-beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide
INTREPID	A randomised, double masked sham controlled multicentre clinical trial to evaluate stereotactic radiotherapy for neovascular age related macular degeneration in patients requiring ongoing intravitreal injection therapy for age related macular degeneration
J/cm^2	joules per centimetre squared
J/cm^2	Joules per square centimetre
Jak	Janus kinase
JNK	c Jun N terminal kinase
Jun-B	protein, encoded by the JUN gene family, component of transcription factor complex

Jun-D	protein, encoded by the JUN gene family, component of transcription factor complex
	labelling
LASER	Light amplification by stimulated emission of radiation
LLUS	Late Leakage of an undetermined source
logMAR	logarithm of minimum angle of resolution
LOX-1	Oxidised low density lipoprotein receptor-1
LPS	lipopolysaccharide
LPS	Lipopolysaccharide
m^2	metre squared
MAC	Membrane attack complex
MAPK	Mitogen activated protein kinase
MARINA	Minimally Classic/ Occult Trial of the Aniti-VEGF Antibody Ranibizumab in the Treatment of Neovascular AMD
MCP-1	Monocyte chemotactic protein-1
MERITAGE	Macular epiretinal brachytherapy in treated age related macular degeneration
MERLOT	UK multicentre randomised controlled trial in patients who have already commenced anti-VEGF therapy. The aim is to compare epimacular brachytherapy and as required ranibizumab with ranibizumab monotherapy.
mg	milligramme
ml	millilitre
ml	millilitres
MMPs	Matrix metalloproteinases
MONT BLANC	A 12 month, randomised, double masked, multicentre study to evaluate the safety and efficacy of standard fluence verteporfin photodynamic therapy in combination with ranibizumab versus ranibizumab and sham verteporfin photodynamic therapy.
MPS	Macular Photocoagulation Study
mRNA	messenger RNA
MTOR	Mechanistic target of rapamycin, a serine/threonine protein kinase part of the phosphatidylinositol-3-kinase related kinase protein family
MTT	3-(4,5-dimethylthiazone-2yl)-2,5-diphenyltetrazolium
MTT	methyl thiazolyl tetrazolium assay
mW/cm^2	milliwatts per centremetre squared
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa beta
NHS	National Health Service
NICE	National Institute for health and clinical excellence
NIK	NF-κB inducing kinase
nm	nanometre
nm	nanometre
NOS	Nitrous oxide synthase

O_2	Oxygen
OCT	Optical coherence tomography
OCT	Optical coherence tomography
OD	Optical density
OD	Oculus dexter, Latin for “right eye”
OS	Oculus sinister, Latin for “left eye”
P13 kinase/Akt	Phosphatidylinositol-3 kinase
P13K	phosphatidylinositol-3-kinase
P2X	family of cation permeable ligand gated ion channels that open in response to the binding of adenosine 5’-triphosphate
P2Y	family of purinergic G protein coupled receptors stimulated by nucleotides such as adenosine triphosphate
p38	cell signalling pathway
	patients with Neovascular AMD Treated with intra-Ocular
PBS	phosphate buffered saline
PC12	cell line derived from phaochromocytoma of rate adrenal medulla
PCR	Polymerase chain reaction
PCV	Polypoidal choroidal vasculopathy
PDGF	Platlet derieved growth factor
PDR	proliferative diabetic retinopathy
PDT	photodynamic therapy
PDT	Photodynamic therapy
PED	Pigment epithelial detachment
pg	picogrammes
PGE2	prostoglandin E2
PI	Propidium iodide
PlGF	Placental growth factor
POS	Photoreceptor outer segments
PRN	pro re nata, Latin for “as circumstances require”
PrONTO	Prospective Optical Coherence Tomography Imaging of
PROTECT	Study assessing the safety of same day ranibizumab administered in conjunction with photodynamic therapy in patients with occult or predominantly classic subfoveal choroidal neovascularisation secondary to age related macular degeneration
PSII	Posterior segment intraocular inflammation
PVR	Proliferative vitreoretinopathy
RAGE	Receptor for advanced glycation end products
	Ranibizumab
RAP	Retinal angiomatous proliferation
REL-A	v-rel avian reticuloendotheliosis viral oncogene homolog A, a member of the nuclear kappa beta family of transcription factors, also known as p65

REL-B	v-rel avian reticuloendotheliosis viral oncogene homolog B, a member of the nuclear factor kappa beta family of transcription factors
ROM1	Retinal outer segment protein 1
ROS	reactive oxygen species
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RPE65	Retinal pigment epithelium-specific protein 65kDa
Rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
S100/granulins	Family of low molecular weight proteins soluble in neutral
SAPK	stress activated protein kinase
	saturated ammonium sulphate
SCI	Spinal cord injury
SD	Standard deviation
SFM	Serum free media
Smac	Second mitochondrial derieved activator of caspases
SNP	Single nucleotide polymorphisms
SRT	Stereotactic radiotherapy
SST	Semisynthetic tetracylines
STAT	transcription pathway
SUMMIT	Clinical development program designed to evaluate the efficacy and safety of verteporfin photodynamic therapy combined with ranibizumab compared with ranibizumab monotherapy in patients with subfoveal choroidal neovascularisation due to AMD
T	time
TAP	Treatment of age related macular degeneration with
TGF-β	Transforming growth factor beta
TIMPS	Tissue inhibitors of matrix metalloproteinases
TMB	3,3’,5,5-tetramethylbezidine
TNF-α	Tumour necrosis factor alpha
	trial
TUNEL	TdT-mediated dTP nick end labelling
TUNEL	Termina l deoxynucleotidyltransferase dUTP nick end
UDP	uridine diphosphate
UK	United Kingdom
USA	United States of America
UTP	uridine 5’-triphosphate
VEGF	Vascular endothelial growth factor
VIM	Verteporfin in minimally classic CNV
VIP	Verteporfin in photodynamic therapy
XIAP	X-linked inhibitor of apoptosis protein
ZO-1	Zona occludens

Chapter 1

1.1 Definition and symptoms of age related macular degeneration (AMD)

Age related macular degeneration (AMD) is an eye condition with onset usually after the age of 50 which progressively destroys the macula, the central portion of the retina, impairing central vision. Early symptoms include blurring of central vision with visual distortion. As central vision is needed for reading, driving, recognising faces and doing detailed work patients generally report difficulties with these tasks. Peripheral vision is usually preserved. The decline to severe loss of vision can vary from months to years depending on the type and severity of AMD.

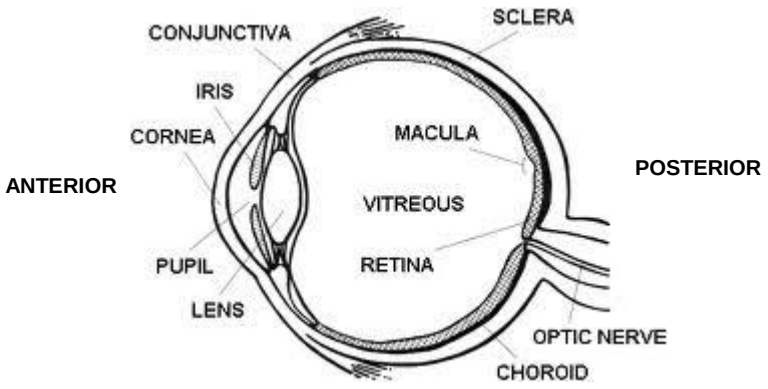

Figure 1: Diagram of the basic anatomy of the eye

1.2 Epidemiology of AMD

At least 1.75 million individuals in the USA suffer from AMD and this figure will rise with the overall ageing of the population. Some estimates have suggested that up to 2.95 million people in the USA may be affected by AMD by the year

2020.[1] In the UK, at least 0.5 million are affected by AMD and 250,000 are registered blind or partially sighted as a result but this may not fully reflect the amount of sight loss as many people who could register chose not to do so.[2] In one study, women had a slightly increased prevalence of AMD compared to men for those over the age of 75.[3] However, this finding contrasts to the results of three pooled studies, the Beaver Dam, Blue Mountain, and Rotterdam Eye Study, where no significant differences in risk were found between men and women.[4] This discrepancy is probably related to the longer life expectancy of women relative to men. Ethnic variations in the prevalence of AMD have also been reported and it has been suggested that this is due to variations in the complement factor H risk allele.[5]

1.3 Macular Anatomy and ageing changes

The retina is the light sensitive tissue at the back of the eye. The retina contains specialised light sensitive cells known as photoreceptors which are responsible for a process known as visual transduction.[6] Visual transduction is a process by which light is converted into electrical signals. Moving from the front towards the back of the eye from the retina several layers of tissues are present consisting of the retinal pigment epithelium, Bruch's membrane, the choriocapillaris and the sclera (Figure 2). The choriocapillaris or choroid layer is a densely vascular structure supplying the outer third of the retina with a blood supply.[6] The macula is an area approximately 5.5mm in diameter located at the centre of the retina. Visual acuity depends on the function of the macula. This is because the macula is the region of the retina with the highest concentration of

photoreceptors, primarily cone cells. At the centre of the macula the fovea is present, an area approximately 1.5mm in diameter. The foveola forms the central floor of the fovea and has a diameter of 0.35mm. It is the thinnest part of the retina and consists only of cones and their nuclei.[6] (Figure 3). The foveal avascular zone is an area devoid of blood vessels at the centre of the fovea. The retinal pigment epithelium (RPE) is a single layer of hexagonal cells, the apices of which manifest villous processes that envelope the outer segments of the photoreceptors. The cells consist of an outer non-pigmented basal element containing the nucleus, and an inner pigmented apical section containing abdundant melanosomes. A melanosome is an organelle containing the light absorbing pigment melanin. At the fovea, RPE cells are taller and thinner, more regular in shape and contain larger melanosomes than in the periphery. The adhesion between the RPE and sensory retina is weaker than between the RPE and Bruch's membrane, which underlies the RPE. The potential space between the RPE and retina is known as the subretinal space. The RPE maintains the integrity of the sub-retinal space by constituting the outer blood retinal barrier, formed by the intervening tight junctional complexes (zonula occludentes). This prevents extracellular fluid, which normally leaks from the choriocapillaris from entering the sub-retinal space and also actively pumps ions and water out of the subretinal space. The RPE cell layer facilitates photoreceptor turnover by the phagocytosis and lysosomal degradation of cone outer segments following shedding. The RPE layer also functions to store, metabolise and transport vitamin A in the visual cycle, the process by which light energy is converted to electrical impulses.[6] Bruch's membrane separates the RPE from the

choriocapillaris and consists of five elements, the basal lamina of the RPE, an inner collagenous layer, a thicker band of elastic fibres, an outer collagenous layer and the basal lamina of the outer layer of the choriocapillaris.

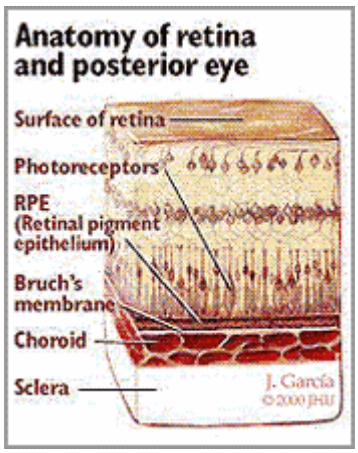

Figure 2: Anatomy of retina and posterior eye (image from whyfiles.org/163amd_eye/images/retina_cross.gif)

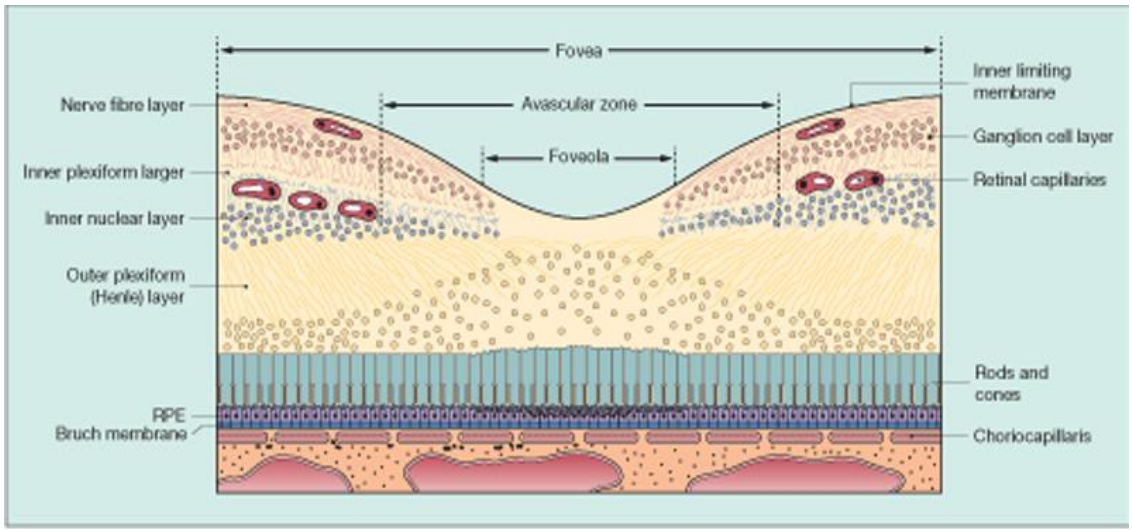

Figure 3: Cross section of the fovea (image from Kanski JJ and Bowling B (2011) Clinical Ophthalmology: A systemic approach. Chapter 14 pg 595)

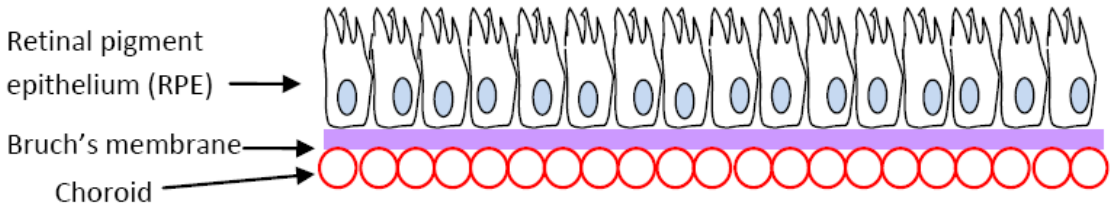

Figure 4: Schematic representation of the normal structural anatomy of the posterior eye

Numerous biological and histological changes take place in the ageing eye and not all of these lead to the development of age related macular degeneration. These changes may evolve concurrently or consecutively and the relationship between them is not entirely clear.

With age, choroidal vascular atrophy occurs and the choriocapillaris loses the ability to transfer oxygen and nutrients to the surrounding cells[7], plasma levels of vitamin C and E decrease [8, 9] and lipid peroxidation increases.[10]

Fine yellow brown pigment granules composed of the lipid residues of lysosomal digestion are known as lipofuscin. It has been reported[11] that lipofuscin accounts for 1% of the RPE cytoplasmic volume in the first decade of life and that this increases to 19% by the 8th decade. Lipofuscin compromises the function of the RPE cells and leads to photoreceptor cell death.

Reactive oxygen species are a product of cellular metabolism and as the photoreceptors and RPE cells account for 90% of the oxygen consumption from

the choriocapillaris the generation of metabolic by-products is high. Light irradiation also stimulates the production of free radicals and leads to oxidative stress and tissue injury. However, it is difficult to quantify the total amount of light exposure throughout life and there is as yet no proven causal relationship between exposure to ultra-violet light and AMD.[12, 13]

The outer segments of the photoreceptors are highly susceptible to oxidative stress. Approximately 50% of the outer membrane is composed of polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA). [14, 15]

The choriocapillaris density decreases with age and the choroidal thickness decreases from 200µm at birth to approximately 80µm at the age of 80.[16] Therefore, with age, the photoreceptors are deprived of the oxygen and nutrients delivered by the choriocapillaris that are required for normal metabolic function. There is a close association between the metabolic processes within the RPE and the photoreceptors.[17] Ischaemia induced damage and dysfunction of the RPE causes secondary degeneration of rods and cones.

Several changes occur in Bruch's membrane with age. Ramrattan and co-workers[16] reported in normal adults the thickness of the membrane increases by 135% (from 2µm to 4.7µm) between birth and the age of 90 years. In eyes that had advanced macular degeneration the density of the choriocapillaris was less than normal for each measurement, as was the choriocapillaris diameter. The thickness of the choroid itself was not different between normal and diseased eyes. Therefore, it was concluded that age was the only factor affecting the

thickness of Bruch's membrane and that it was not connected to the age related atrophy of the choriocapillaris.[16]

1.4 Pathophysiology of AMD

Risk factors for AMD include increased age, smoking, diet, light exposure and studies have linked genetics, oxidative stress, RPE senescence, hypoxia and other factors.[17-23] Age is the major risk factor and family history is important. The risk of developing AMD is greatly increased by having an affected first degree relative.[23] Variants in many genes have been implicated in AMD risk and protection such as chromosome 1q32 for complement factor H (CFH), which protects cells from complement mediated damage, the hemicentin gene on 1q24-25, and the ABCR gene on chromosome 1p. The ABCR gene is also implicated in other retinal conditions such as Stargardt disease and fundus flavimaculatus.[6] Smoking is considered to approximately double the risk of AMD and hypertension and other cardiovascular risk factors are likely to be associated.[6] There is substantial evidence from the AREDS[24] study that taking high dose antioxidant vitamins and minerals on a regular basis can decrease the risk of AMD progression. The regimen used in AREDS included 500mg vitamin C, 400IU of vitamin E, 15mg of beta-carotene and 80mg of zinc, with 2mg of copper (cupric oxide) to prevent zinc induced copper deficiency. The reduction in risk of progression to further visual loss at 5 years is in the order of 25% for those taking supplements with the more advanced of these signs at

baseline. Supplements did not discernibly reduce progression in those with early or no AMD.[24] More recently, the AREDS 2 clinical trial examined whether lutein and xeaxanthin and omega 3 long chain polyunsaturated fatty acids (DHA and EPA) were associated with a reduced risk of advanced AMD in addition to the original AREDS formulation. [25] The secondary goal of the AREDS 2 study was to test the effects of eliminating beta carotene and zinc from the original AREDS formulation. The results of the AREDS 2 study[25], a prospective randomised trial conducted over a 5 year period showed no statistically significant effect of additional supplements in addition to the original AREDS formulation.

However, although antioxidants appear to reduce the risk of progression of AMD, the exact aetiology and pathogenesis of AMD remains largely unclear. An increasing collection of evidence suggests that immunologic events play a key role in the pathogenesis of AMD.

Ocular inflammation associated with uveitis manifests with inflammatory cell infiltrate, oedema and tissue damage. In AMD, the inflammatory process is less overt and may link the risk factors together finally leading to visual loss. The inflammatory process results from interactions between various immune components.

1.5 Inflammation in AMD

AMD is predicted to become more prevalent worldwide as life expectancy continues to increase. The pathogenesis of this condition is incompletely

understood and chronic oxidative stress has been recognised recently as an important contributory factor in the pathophysiology.[19] The evidence for the role of inflammation in the aetiology of neovascular AMD will be discussed before considering a therapeutic role for minocycline.

1.5.1 General aspects of inflammation

Inflammation is the response of a tissue to a noxious stimulus.[26] Inflammation is usually described as several phases. In the acute phase, the classic cardinal signs of redness (rubor), heat (calor), mass (tumor), pain (dolor) and loss of function are usually described. These are caused by an increased rate and volume of blood flow, exudation of fluid (oedema) and cells and irritating chemicals. Chemical mediators increase vascular permeability by causing the usually “tight” junctions between adjacent vessel endothelial cells to open, allowing fluid to leak into the surrounding tissue spaces. The subacute phase of inflammation varies greatly and is concerned with the healing response.[26] The outcome of this phase may be restoration of normal haemostasis, scarring, or chronicity of the inflammatory response. The chronic phase of inflammation involves specific cellular responses. The inflammatory response is complex involving a multitude of chemical mediators and a variety of cells and pathways. The foregoing is not intended as an exhaustive review of the inflammatory response. The evidence for inflammation and the immune response in the context of the aetiology of AMD will be discussed.

1.5.2 Infection and inflammation in AMD

Evidence for the role of inflammation in the aetiology of AMD arises from links with past infections and the condition. Chlamydia pneumonia is an obligate intracellular pathogen that can give rise to persistent asymptomatic infection, resulting in chronic inflammation.[27] Chlamydia pneumonia infection results in an activation of pro-inflammatory pathways some of which are also implicated in AMD. The alternative pathway of complement that is strongly implicated in AMD pathogenesis can be activated by Chlamydia. Infection with this pathogen may lead to complement overactivity in patients with the Y402H variant of complement factor H (CFH) that leads to impaired complement inhibitor function by CFH. AMD has been associated with macrophage accumulation, and studies have shown that Chlamydia infection promotes differentiation of monocytes to macrophages.[28] Chlamydia infected monocytes exhibit enhanced migration through the blood brain barrier [29] and this poses the question whether a similar process may occur through the blood retinal barrier.

Two studies [30, 31] showed serum antibodies for Chlamydial pneumonia proteins are associated with increased risk of AMD development and progression. In vitro, macrophages produce vascular endothelial growth factor (VEGF) after infection with Chlamydia pneumonia.[32] However, further studies [33, 34] failed to demonstrate an association between Chlamydia pneumonia and AMD. Further studies are needed to elucidate the potential patho-physiological connection between Chlamydia infection and AMD.

1.5.3 Inflammation, the complement system and AMD

In patients with posterior segment intraocular inflammation (PSII) the incidence of choroidal neovascularisation (CNV) varies with different types of inflammation. CNV is a recognised feature of PSII conditions such as multifocal choroiditis with panuveitis, punctuate inner choroidopathy, serpiginous choroiditis, posterior sympathetic ophthalmia, angioid streaks, ocular toxoplasmosis and pathological myopia.[35] Chronic inflammation and angiogenesis are linked together not just in retinal disorders but also in other conditions such as rheumatoid arthritis, diabetes, and cancer. In rheumatoid arthritis, new blood vessels are formed during early stages of inflammation, increasing leukocyte movement into the synovium [36] where leukocyte–endothelial interaction may play an important role in initiation and progression of synovial angiogenesis.[37] During cancerogenesis, the ischemic microenvironment and tumour-associated macrophages are central for tumour angiogenesis.[38] In the retina, however, detailed mechanisms on how angiogenesis is formed (either from the choroid or *de novo* from the retina) in the presence of chronic inflammation remain poorly defined.[35]

Immunity is defined as the ability of a host to protect itself against a foreign antigen. Host defence mechanisms in humans against pathogens take the form of an innate system which is non specific and rapidly mobilised. This comprises a first line of defence involving a physical barrier to entry such as eyelids and tears, molecules such as complement, phagocytic/ cytotoxic cells such as natural killer cells and macrophages and cytokines such as the interleukins and

tumour necrosis factor α (TNF). The adaptive immune response includes cells (lymphocytes) with receptors that specifically recognise foreign organisms and molecules (antigens) and specific molecules (antibodies) which specifically counteract foreign antigen.

The complement system comprises several soluble and membrane bound proteins, and can be divided into three main pathways: classical, lecithin and alternative.[39] The classical pathway is activated largely by immune complexes (antibody bound to antigen); the lecithin pathway is activated primarily by mannose and N-acetyl glucosamine residues that are particularly abundant on bacterial cell surfaces, and the alternative pathway is initiated by a variety of activating substances including microbial surfaces and polysaccharides. Activation of these pathways results in a pro-inflammatory response including generation of membrane attack complexes (MAC) which mediate cell lysis, release of chemokines to attract inflammatory cells to the site of damage, and enhance capillary permeability [39,40]. Intraocular complement regulatory proteins tightly control the process of complement regulation to ensure elimination of potential pathogens without damage to healthy tissue. Complement dysregulation has been well characterized in autoimmune anterior uveitis [41] and may be implicated in AMD pathogenesis.

The complement component C5 and components C5b-9 forming the membrane attack complex have been identified in drusen from human eyes, including eyes with AMD.[42] Also C3a and C5a have been localized to drusen, RPE cells and Bruch's membrane in human AMD.[43] Recently, genetic analyses for single

nucleotide polymorphisms (SNP) spanning the complement genes C3 and C5 has linked the arginine to guanine at amino acid 80 (R80G) SNP in the C3 gene to AMD.[44]

Complement factor H (CFH) is a regulator of the complement system. It impairs activation of the alternative pathway by inhibiting several steps and by promoting degradation of activated complement components. CFH binds heparin on cell surfaces to prevent complement mediated damage to heparin. It also binds C- reactive protein (CRP) to inhibit CRP mediated activation of the alternate pathway in response to damaged tissue.[45] CFH is expressed in a variety of human ocular tissues, including sclera, pigment epithelium, retina and choroid. Studies in mice[46] demonstrated that ocular expression of CFH increases with age. Hence CFH might accumulate with age to inhibit alternate pathway activation and impairment in CFH function might create a pro-complement, that is, pro-inflammatory environment leading to AMD. Several studies have reported that the tyrosine to histidine SNP at position 402 (Y402H) in the CFH gene in the 1q32 region increases the risk of AMD and a meta-analysis by Thakkinstian[47] suggested that up to 50% of all AMD is associated with this polymorphism.

The link between CFH and AMD has been further strengthened by a variety of in vitro studies. Oxidative stress has been associated with AMD and long term treatment of RPE cells with oxidized photoreceptors, but not non-oxidized photoreceptors impairs CFH secretion. The presence of pro-inflammatory cytokines such as IL-6 also decreases CFH secretion by RPE cells.[48] Hence it

is also possible that not only genetic variation in CFH but also processes such as oxidative stress which impair CFH synthesis and/ or secretion may lead to overaction of the alternative pathway and AMD pathology. Deficiency or impaired function of CFH is also associated with membranoproliferative glomerulonephritis type 2, a renal condition which is associated with deposits that may result from a similar inflammatory process as that which produces drusen seen in AMD.[49]

Macrophages and chemokines that mediate macrophage recruitment to the retina have also been associated with AMD. One chemokine that has generated particular interest is CCL2 and its receptor CCR2. CCR2 is a chemokine receptor found on macrophages which binds CCL2 chemokine. Deficiencies in CCR2 lead to decreased leukocyte adhesion to microvasculature as well as decreased extravasation of monocytes from the circulatory system into surrounding tissue. As yet, there have been no direct studies of CCL2 or CCR2 in human AMD. However, altered expression of CCR2 has been implicated in studies of apolipoprotein E (ApoE) polymorphism in human AMD. Bojanowski [50] demonstrated that the Apo E 112R variant is associated with decreased risk of AMD by decreasing expression of CCL2 in RPE cells and therefore reducing macrophage recruitment to the retina. This suggests macrophages are involved pathologically, rather than in a restorative role in AMD.

However, a study of CCL2 and CCR2 deficiency in a knockout mouse animal model suggested macrophages may serve a beneficial role in the retina.[51]

These deficient mice developed AMD like lesions starting at 9 months of age, histologically these lesions consisted of lipofuscin granules, and thickening of Bruch's membrane. By the age of 16 to 18 months these mice developed macula atrophy and choroidal neovascularisation. The study [51] also demonstrated that drusen components such as C5, IgG and glycosylation products upregulate CCR2 expression by RPE cells and choroidal endothelial cells. The study [51] showed in vitro, that macrophages adhere to IgG and C5 and facilitate their clearance. At present it is still unclear whether macrophages contribute to the pathology of AMD as suggested by limited human studies or whether they accumulate as an adaptive response.

Grossniklaus [52] and co- workers derived a possible mechanism whereby macrophages may promote choroidal neovascularisation (CNV). They [52] showed that macrophages express the pro-angiogenic vascular endothelial growth factor (VEGF) so may directly promote the formation of CNV.

Kamei et al [53] suggested another plausible function of macrophages at sites of CNV lesions. They [53] demonstrated that CNV membranes contain oxidized lipoprotein and that these membranes stain positive for macrophages bearing scavenger receptors for oxidized proteins including lecithin like oxidized low density lipoprotein receptor-1 (LOX-1). The study [53] suggests that macrophages seen in AMD may ingest oxidized low density lipoprotein that accumulates with age. This emphasized similarities with the mechanism in atherosclerosis and suggested macrophages play a pathologic role in CNV. Overall, although these studies[52, 53] suggest a role for macrophages in CNV, they do not prove a

causative role. This is largely due to the limitations of human studies where it is impossible to manipulate macrophage numbers to determine whether they increase or decrease the likelihood of CNV formation. Research is therefore confined to animal models for CNV formation.

There are myeloid derived cells in the retina, called microglial cells. These cells have been implicated in ocular inflammatory disease such as uveitis and they may have a role in the pathogenesis of AMD. In vitro studies involving treatment of healthy photoreceptors with activated microglia suggest that this may induce injury to healthy photoreceptor cells.[54] The microglia chemokine receptor CX3CR1 has been studied in human and animal models of AMD. This receptor is G-protein coupled and has been found on inflammatory cells, including microglia, macrophages, astrocytes, and T cells. The ligand CX3CL1 is a chemokine that binds to CX3CR1 receptors on leukocytes to promote activation of the leukocytes and mobilization of these leukocytes to sites of inflammation.[55] CX3CR1 is expressed in the retina where it may mediate influx of microglia and macrophages to clear accumulated deposits.[56]

Combadiere and co-workers [57] demonstrated that CX3CR1 positive cells in the outer retina and subretinal space of human AMD eyes, in drusen and near choroidal neovasculization are microglial cells. They showed CX3CR1 deficient mice exhibited signs of retinal degeneration.[57] The microglia formed drusen like deposits between the photoreceptor outer segment and RPE and similar deposits were identified in human AMD eyes.[57]

1.5.4 Role of growth factors in age related macular degeneration

Growth factors, neurotrophic factors, chemokines and cytokines are cell secreted mediators of autocrine and paracrine functions, involved in cell maintenance, survival, growth and death. They are also involved in angiogenesis, vascular permeability, and inflammation.[58] Vascular endothelial growth factor (VEGF), identified as being important in angiogenesis, is now thought to have a role as mediator of leukocyte adhesion in vascular cells and as a neurotrophic factor in the central nervous system.[58] Inhibition of VEGF is now in widespread use for treating exudative AMD. However, inhibition of angiogenesis alone is not sufficient to restore macula function in AMD if in addition to CNV the clinical and pathological spectrum of AMD shows a variety of changes, ranging from drusen deposition and pigment epithelium detachments to scar formation.

1.5.5 Growth factors in AMD pathogenesis

Many growth factors have been implicated in the pathogenesis of AMD, based on experimental data obtained from studies in cultured retinal pigment epithelium (RPE) and from histopathological studies in surgically removed human CNV or in laser induced CNV in transgenic rodent models.[60,61] These studies identified the RPE as an important source of growth factors involved in tissue maintenance, homeostasis, inflammation and CNV formation and also as the central cell type involved in regulating these processes (Table 1). This is of

particular relevance for research exploring transplantation of the RPE as a therapeutic approach in AMD.

Table 1 Growth factors and cytokines: functions in choroidal neovascularization[62]

Process	**Growth factors involved**
Neutrophil infiltration	TGF-β, MCP-1
Macrophage infiltration	TGF-β, MCP-1
Angiogenesis	VEGF-A, PLGF, FGF2, angiopoietins, HGF, MCP-1
Fibroplasia	PDGF, TGF-β, CTGF, IGFs
Matrix deposition	FGF2, IGF-1, TGF-β, MCP-1, CTGF
Scarring	IGF-1, TGF-β, CTGF
Re- epitheiliaization	FGF2, IGFs, MCP-1, TGF-β

1.6 Clinical features of AMD

The clinical features of AMD were first described by Needelship in 1884 as a central choroidal atrophy.[63] It was Haab in 1885 who used the term senile macular degeneration to describe the condition.[64] The earliest clinical manifestation and pathological feature of AMD is the development of drusen, extracellular deposits of glycoproteins, lipids and cellular debris located inside Bruch's membrane and beneath the RPE. A few small drusen can be found in healthy individuals over the age of 50, however, the presence of large or numerous drusen confers significant risk for AMD. [18, 19] Hard drusen are well defined and less than half a retinal vein width (<63µm) in diameter. Their presence as the only finding probably carries little increased risk of visual loss. Soft drusen are less distinct and generally substantially larger than hard drusen. It has been suggested that the presence of more than five soft drusen might be taken as the defining feature of age related maculopathy (ARM) [6] As soft drusen enlarge and become more numerous, they may coalesce giving a localised elevation of the RPE, a "drusenoid RPE detachment". Dystrophic calcification may develop in both types of drusen (figure 5).

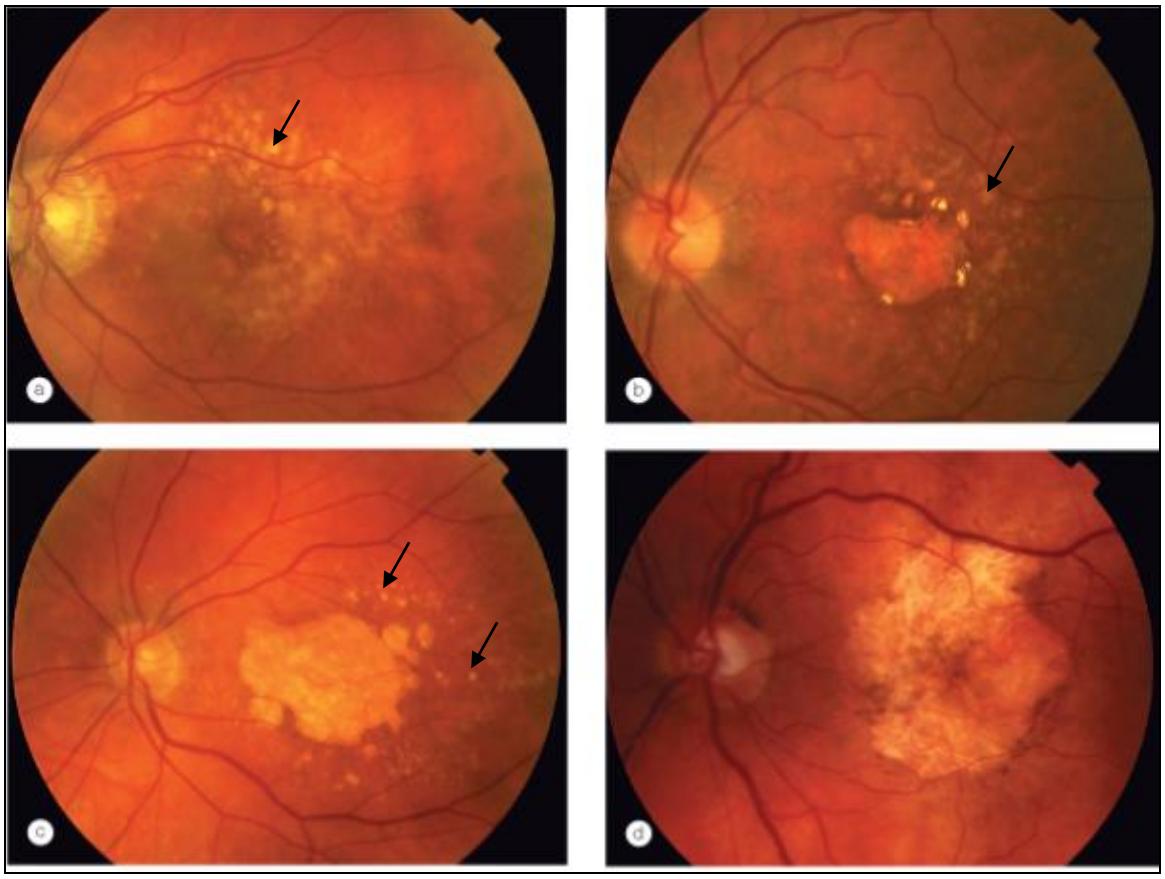

Figure 5: Atrophic age related macular degeneration (a) Drusen and mild RPE change (b) Drusen and moderate retinal atrophy (c) Drusen and geographic atrophy (d) geographic atrophy and disappearance of drusen (black arrows point to the drusen)
(image from Kanski JJ and Bowling B (2011) Clinical Ophthalmology: A systemic approach, chapter 14, pg.617 pub Elsevier Saunders)

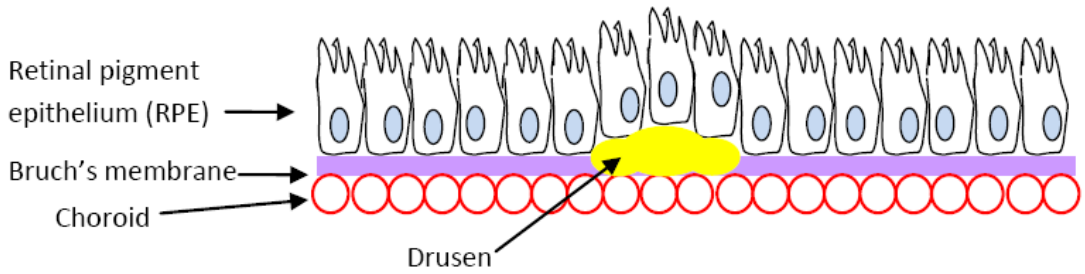

Figure 6: Schematic representation of the structural anatomy of the posterior eye in a patient with drusen and dry age related macular degeneration

Clinically and histologically, AMD is generally classified into two major sub-types: dry and wet. Choroidal neovascularisation (CNV) is a characteristic of the wet form, accounting for approximately 10% of cases.[19] Dry AMD progresses more slowly and manifests with drusen, geographic atrophy of RPE and photoreceptor dysfunction and degeneration. Wet AMD is more debilitating and often presents after dry AMD. Choroidal neovascularisation (CNV) is the growth of new vessels from the choroid into the region underlying the RPE or extending past the RPE into the subretinal space and retina (figure 7).This choroidal neovascularisation can lead to leakage of blood into the subretinal space, which combined with RPE atrophy and photoreceptor degeneration, leads to visual loss. Fluorescein angiography (FA) is used primarily to confirm a suspected diagnosis of CNV prior to committing the patient to anti-VEGF treatment. Fluorescein angiography is a technique for examining the circulation of the retina using the dye tracing method. It involves an injection of sodium fluorescein into the systemic circulation, and then an angiogram is obtained by photographing the fluorescence emitted after illumination of the retina with blue light. A barrier yellow filter in the camera allows only light from the fluorescence to reach the camera. The terminology used to describe the FA appearances of CNV is derived from the Macular photocoagulation study (MPS). [65]

Classic CNV is a well defined membrane which fills with dye in a ‘lacy’ pattern during the early phases of dye transit, then leaks into the sub-retinal space and around the CNV over 1-2 minutes (Figure 8). Classic CNV is classified topographically according to its relation to the centre of the foveal avascular

zone (FAZ): Extra-foveal; 200-1500µm from the centre, Juxta-foveal; 1-200µm and sub-foveal. Most membranes are sub-foveal at presentation.[6]

Occult CNV is used to describe CNV when its limits cannot be fully defined on FA, typically when growth is between the RPE and Bruch's membrane. Variants distinguished in the MPS classification [65] are fibrovascular pigment epithelial detachment (PED) and 'late leakage of an undetermined source' (LLUS), areas of leakage in the late phase of the angiogram without classic CNV or fibrovascular PED. CNV may be said to be 'predominantly' or 'minimally' classic when the classic component is greater or less than 50% of the total lesion size.

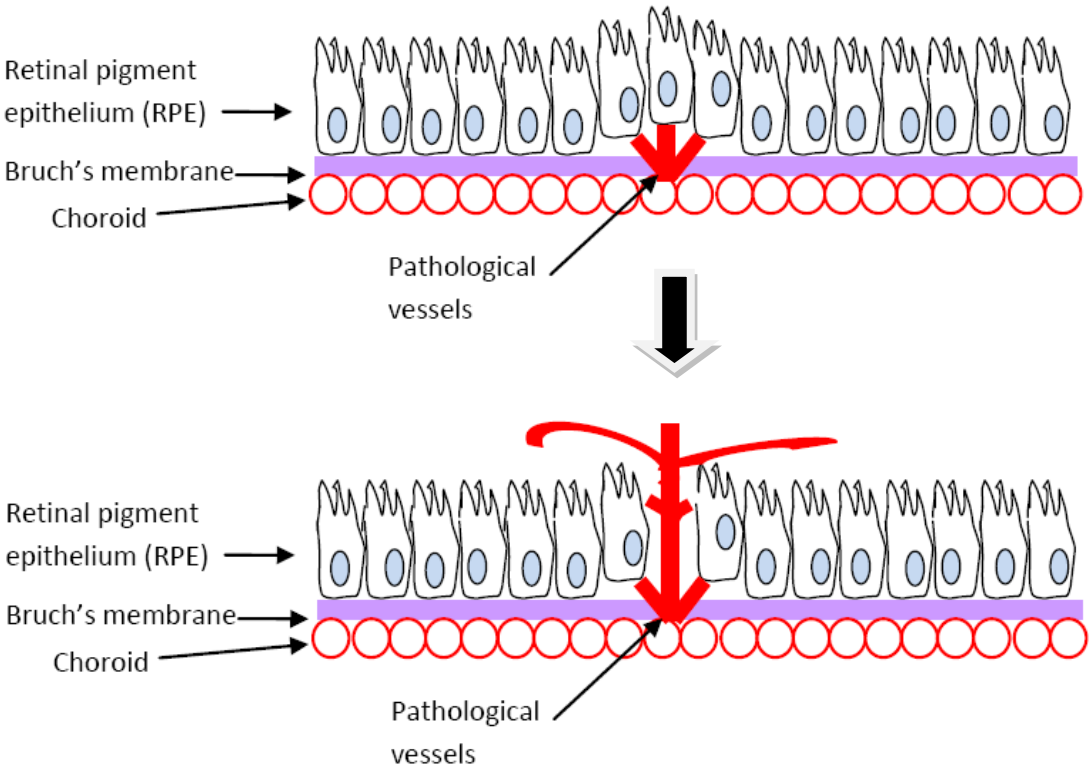

Figure 7: Schematic illustration of progression of pathological neovascularisation associated with wet AMD

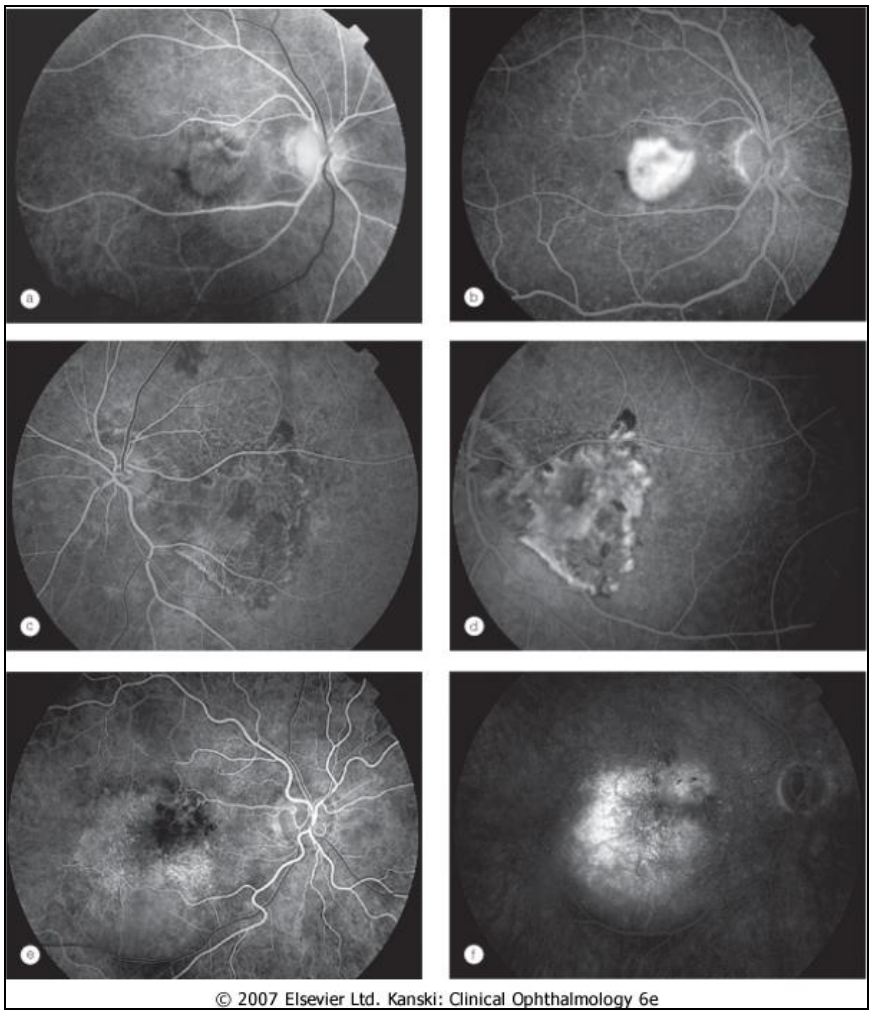

Figure 8: FA of choroidal neovascularization. (a and b) Classic subfoveal; (c and d) very large subfoveal; (e and f) occult (image from Kanski J(2007) Clinical Ophthalmology: A systemic approach Pub Elsevier Saunders)

1.7 Treatments for AMD

The goal of AMD treatment is to delay or reduce the risk of visual acuity loss. There is still a high incidence of persistence and recurrence of CNV shown in histopathological studies of excised, previously treated CNV membranes.[62]

1.7.1 Laser

Lasers are devices that emit light (electromagnetic radiation) based on stimulated emission of photons. The term “laser” originated as an acronym for light amplification by stimulated emission of radiation.[66] Photocoagulation is the process by which clotting of tissue occurs on exposure to laser energy that is selectively absorbed by haemoglobin, the pigment in red blood cells, in order to seal leaking blood vessels.

During the 1980s and 1990s the Macular Photocoagulation Group (MPS) [65] established the efficacy of thermal laser photocoagulation to treat selected patients with neovascular age related macular degeneration. In controlled studies the MPS group showed that laser photocoagulation slowed the loss of visual acuity in selected patients with CNV secondary to AMD.[65] The likelihood of success with laser photocoagulation was affected by initial visual acuity, CNV type, size of the lesion, and the location of the lesion relative to the fovea. As a result of these findings, laser photocoagulation is recommended only for patients with well defined extra-foveal or juxta-foveal CNV.[67, 68] Additionally, patients with small sub-foveal lesions can be treated if the lesion is well demarcated and has evidence of classic CNV.[69] Application of these eligibility criteria in clinical practice has shown that laser photocoagulation is a suitable treatment for about 13 to 26% of patients with neovascular AMD.[67] Laser photocoagulation has its limitations. Treatment of sub-foveal lesions by laser therapy destroys the retinal tissue overlying the lesion and may result in an immediate loss of visual acuity and irreversible loss of visual function. A high

rate of recurrence of CNV after thermal laser photocoagulation has been reported. Over 50% of laser treated eyes have persistent CNV or recurrent CNV within 3 to 5 years of treatment and this is usually associated with further deterioration of vision. [68-70]

1.7.2 Photodynamic Therapy

Verteporfin therapy is a two step process in which the drug is first administered by intravenous infusion and then activated in the target area by red light at a specific wavelength (689nm) by a non thermal diode laser. Activation of verteporfin leads to the formation of singlet oxygen that disrupts endothelial cells in the CNV target tissue, and induces platelet aggregation, photothrombosis, vascular occlusion, stasis of blood flow, and eventual eradication of CNV.[71-74]

Verteporfin (visudyne) photodynamic therapy has been shown to inhibit leakage of blood and fluid from CNV [71] and has been used as an effective alternative to thermal laser therapy. The approval of verteporfin for subfoveal, predominantly classic CNV due to AMD in 2000 by the Food and Drug administration (FDA) in the USA represented a significant breakthrough. The safety and efficacy of verteporfin monotherapy has been demonstrated in approximately 1000 patients enrolled in the Treatment of age related macular degeneration with photodynamic therapy (TAP) investigation, Verteporfin in photodynamic therapy (VIP) trial and Verteporfin in minimally classic CNV (VIM) trial. [72-77] Visual acuity benefits of verteporfin over placebo were sustained throughout 1 to 2 year

follow up.[75, 76] The durable effects of verteporfin on vision and contrast sensitivity outcomes were demonstrated in the 5 year TAP extension[75] and no new safety concerns or evidence of cumulative toxicities from repeated verteporfin therapy were raised.[75, 76] Between 0.7% and 4.9% of patients treated with verteporfin in the TAP and VIP AMD trials developed acute severe visual acuity loss. [76] This was defined as a decrease in visual acuity of ≥ 20 letters within 7 days of therapy. However, it has been suggested that this does not outweigh the benefits of therapy.[77] One of the advantages of verteporfin therapy is that it offers a finite course of therapy. Yearly treatment rates declined from 3.5 treatments in the first year to 1 in the fifth year.

1.7.3 Verteporfin in combination with anti-inflammatory agents

In addition to robust anti-inflammatory effects, corticosteroids have antiangiogenic, antifibrotic and antipermeability properties.[78, 79] Corticosteroids are potent inhibitors of neovascularisation, and have been shown to act on the neovascular cascade by directly suppressing levels of VEGF.[80, 81] Combination therapy of verteporfin with intravitreal triamcinolone appears to be associated with stabilization of visual acuity rather than sustained improvements. [82, 83] Intravitreal triamcinolone is associated with increased intraocular pressure [84] and cataract progression,[85] it is still considered a therapeutic option if patients are monitored regularly and prescribed glaucoma medication if necessary.

1.7.4 Anti-VEGF treatment

Angiogenesis, which is the growth of new blood vessels from existing vasculature is associated with loss of visual acuity in various ocular conditions[86] In neovascular AMD growth of abnormal blood vessels in the macula region leads to loss of vision. Early events in choroidal neovascularisation (CNV) development such as oxidative stress and ischaemia promote the production of vascular endothelial growth factor (VEGF) which is a key component of the pathogenic process leading to development of CNV.[87-89] The main aims of anti-VEGF therapies are to inhibit cell proliferation, reduce the formation and growth of new blood vessels and minimize vascular leakage.

In 1948 Michaelson [90] postulated that a diffusible substance produced by the retina was responsible for retinal and iris neovascularisation. In 1992 Shweiki[91] et al demonstrated hypoxia induced VEGF mRNA expression in cell culture. Aiello et al [92] confirmed VEGF levels in ocular fluid correlated with neovascularisation secondary to diabetic retinopathy and ischaemic retinal vein occlusion. In 1996 Kvanta et al [93] confirmed a role for VEGF in choroidal neovascularisation. Since initial discovery of the VEGF gene (now known as VEGF-A) several closely related genes have been identified including VEGF-B, VEGF-C, VEGF-D and PIGF (placental growth factor). VEGF-A signalling represents the rate limiting step in normal and pathogenic angiogenesis.[94] The VEGF-A gene consists of several different isoforms.[95] VEGF-165 is the predominant isoform and the primary mediator of neovascularisation in the eye.[96]

The primary trigger of VEGF-A gene expression in the eye is hypoxia. [97,98] Hypoxia inducible factor (HIF-1) a transcription factor, is a key player in hypoxic responses in the cell [98] and is the primary mediator of hypoxia induced gene expression of VEGF-A.

VEGF exerts multiple effects on endothelial cells relating to its function as a pro-angiogenic factor. These include stimulation of cell proliferation, invasion, migration and enhancement of cell survival. The mitogenic effects of VEGF are mediated through protein C and MAPK (mitogen activated protein kinase) pathways. Endothelial cell survival is mediated by the phosphatidylinositol-3-kinase (PI3 kinase/Akt pathway), and also by upregulation of anti-apoptotic proteins Bcl-2, and XIAP. Cell invasion and migration are mediated through upregulation of integrin expression, alteration of the cytoskeleton and induction of metalloproteinases.[96, 99] VEGF has a potent effect on vascular permeability and this is of particular importance in the pathogenesis of choroidal neovascularisation. Vascular leakage is considered to facilitate angiogenesis because the leakage of plasma proteins and fibrin creates a gel like environment conducive to endothelial cell growth and migration. Increased permeability is a result of both vasodilatation and an uncoupling of endothelial tight junctions. VEGF induced increased vascular permeability seems to be mediated in part via the nitrous oxide synthase (NOS) pathway[100] and this may explain why hypertension has been observed in some patients treated with VEGF inhibitors.

Pegaptanib (Macugen; OSI/Eyetech Pharmaceuticals, New York, USA) was the first anti-VEGF agent to be approved for neovascular AMD, conferring greater efficacy compared with sham injections.[101] Despite this, a mean decline in visual acuity of up to 10 letters was observed at 54 weeks in patients receiving 0.3 to 3.0mg pegaptanib.[101] Pegaptanib has proved less efficacious than current treatments, possibly due to selective binding of VEGF-165 and is no longer used in most countries.[102]

Subsequently, ranibizumab was developed and is the first therapy for neovascular AMD to result in significant improvement in visual acuity.[103] Ranibizumab (Lucentis; Genentech, San Francisco, CA, USA) was approved by the Food and Drug Adminstration (FDA), USA in June 2006 for use in the treatment of CNV secondary to age related macular degeneration. The fragment antigen binding (Fab fragment) is a region on an antibody that binds to antigens. The fragment crystallizable (Fc region) is the region of an antibody that interacts with cell surface receptors called Fc receptors and proteins of the complement system. Ranibizumab is a humanized, affinity maturated Fab fragment of a murine monoclonal antibody directed against human VEGF-A. As ranibizumab lacks the Fc region of a full length antibody it is less likely to incite an immune response as it is unable to bind to the complement C1q or Fc receptors.[94] Its small size allows efficient penetration through the retina after intravitreal injection to reach pathologic blood vessels that are being formed in the retinal pigment epithelium-choroid complex.[104] A randomized controlled trial[105] in 716 patients with CNV due to AMD showed that after 12 months, 95%

of patients treated with monthly intravitreal ranibizumab (0.5mg) lost < 15 letters compared to 64% of those receiving placebo ($p < 0.001$). More patients receiving ranibizumab had improvements in visual acuity of ≥ 15 letters compared to placebo recipients (34% versus 5%; $P < 0.001$) and benefits in visual acuity were maintained at 24 months.[105] Ranibizumab was also shown to be superior to verteporfin in the treatment of 423 patients with predominantly classic neovascular AMD. The results[106] showed approximately 95% of those treated with ranibizumab lost < 15 letters at 1 year, compared to 64% in the verteporfin group ($P < 0.001$).[106]

Bevacizumab (Avastin; Genentech) is a full length humanised murine monoclonal antibody directed against human VEGF-A. It was approved by the FDA in 2004 for the intravenous treatment of metastatic colorectal cancer. Its potential for the treatment of CNV was first tested by Michels et al[107] via intravenous infusion in a 12 week uncontrolled study. Positive effects were observed on both visual acuity and optical coherence tomography (OCT) and angiographic characteristics of neovascular lesions. Patients experienced a mean increase of blood pressure of 12mmHg in systolic blood pressure which was felt to be a deterrent to use of this systemic therapy for neovascular age related macular degeneration. However, the promising visual and anatomic results led investigators to consider intravitreal injection of bevacizumab.[108] Several short term, uncontrolled, non-randomized studies have demonstrated that bevacizumab has activity in patients with sub-foveal CNV secondary to AMD. Anatomic improvements in retinal thickness were accompanied by

increases in visual acuity.[108-112] Intravitreal administration of bevacizumab has not resulted in systemic side effects of hypertension in any of these studies. The systemic concentration of bevacizumab after intravenous administration is several times larger than the systemic concentration seen after intravitreal injections and this may account for this observation. No specific toxicity associated with bevacizumab use has been demonstrated at the present time. However, the lack of large scale trials to establish safety have limited its use and it remains an unlicensed treatment for neovascular age related macular degeneration in the UK.

Afilbercept is a novel anti VEGF drug developed by Regeneron Pharmaceuticals Inc. (Tarrytown, NY, USA). Structurally, Afilbercept is a fusion protein of key binding domains of VEGF receptors 1 and 2 combined with a human IgG Fc fragment.[113] Afilbercept is designed to inhibit placental growth factors 1 and 2, in addition to all isoforms of VEGF-A.[113] In November 2011 the drug was approved by the FDA for use in neovascular AMD in the USA. The drug was approved by the national institute for health and clinical excellence (NICE) in July 2013 for wet AMD meaning that it is available for NHS patients.

1.7.5 Factors limiting anti VEGF monotherapy in AMD

The use of antiVEGF therapy has revolutionised the treatment of neovascular age macular degeneration, a condition which previously was largely untreatable and a significant cause of sight loss in the elderly. Despite the positive benefits

of therapy in maintaining and improving vision in patients, several factors may limit the use of anti-VEGF monotherapy that will now be discussed.

Anti VEGF monotherapy does not improve vision in all patients and it appears frequent retreatment is necessary to maintain efficacy.[105,106] In the two pivotal clinical trials establishing efficacy of treatment approximately two thirds of patients did not gain ≥ 15 letters at 12 months after treatment with ranibizumab.[105,106] The cost of treatment and attendance for monthly treatment and follow up for many elderly patients led to studies to determine whether a less frequent dosing regimen was as effective as monthly intravitreal therapy.

In one such study[114] patients received ranibizumab monthly for 3 months followed by therapy quarterly thereafter. This provided improved visual acuity benefits compared with placebo at the 12 month follow up although the treatment effect declined in the ranibizumab groups during the quarterly dosing phase.[114] The decreased treatment effect was accompanied by an increase in vascular leakage and mean retinal thickness during the quarterly dosing regimen suggesting some patients needed ranibizumab injections more frequently to control neovascular leakage.[114]

VEGF inhibition alone although successful in reducing the level of sub-retinal fluid associated with CNV and helping prevent early CNV development is unlikely to have an impact on CNV pathogenesis once the vasculature becomes more established.[115,116] In experimental models, anti-VEGF therapy becomes less effective at inhibiting vessel growth and regressing vessels as

neovascularisation develops over time. [116, 117] Hence, it is unlikely that complete and sustained resolution of CNV will be achieved with anti-VEGF therapy alone. Intravitreal injections merely control active leakage from the CNV and do not treat the underlying pathological process. Therefore, a rationale exists for a combination therapeutic approach targeting different processes involved in CNV.

Currently, patients are subjected to a rigorous treatment regimen incorporating imaging and monthly intravitreal injections with no defined end point. Long term patient compliance is also likely to be affected by a high frequency of injections and some patients may be considered unsuitable for this type of treatment, including those with a chronic ocular or periocular infection. Induction maintenance anti VEGF regimens, comprising an induction phase of three injections at monthly intervals followed by injections as needed depending on assessment of disease activity based on OCT thickness is now in widespread use, following the protocol established in the prospective OCT imaging of patients with neovascular AMD treated with intraocular ranibizumab study (PrONTO) study.[118] The aims of this study were to investigate after an induction phase of three consecutive monthly intravitreal ranibizumab injections whether an OCT guided treatment regimen could be used to maintain improvements in visual acuity over 2 years. However, the burden in terms of frequency of monthly clinic attendances still remains significant despite a reduction in the number of intravitreal injections administered. Sustaining improvements in

visual acuity associated with controlling disease activity with fewer treatments is a goal of combination therapy.

Although ranibizumab is generally well tolerated, it has been associated with relatively infrequent serious ocular adverse events such as endophthalmitis. The incidence of endophthalmitis with ranibizumab was reported at 0.03% following 6,347 injections performed as an office based procedure.[119]

The concern that anti VEGF blockage may lead to increased occurrences of stroke and cardiovascular events in patients has implications for long term use of intravitreal anti-VEGF therapies.[120] Serious non ocular adverse events including myocardial infarction and cerebral vascular events were reported in <5% of patients in the MARINA and ANCHOR trials.[105, 106] Recently, the comparison of age related macular degeneration treatments trial (CATT) has been published that aimed to assess the relative safety and efficacy of ranibizumab and bevacizumab and to determine whether an as needed regimen would compromise long term visual acuity as compared with a monthly regimen.[121] The results of this trial demonstrated that the two drugs were comparable in efficacy when administered in identical regimens. However, mean decrease in central retinal thickness was greater in the ranibizumab administered monthly group than in other groups. Rates of death, myocardial infarction and stroke were similar for patients receiving either bevacizuamab or ranibizumab. The proportion of patients with serious systemic adverse events (primary hospitalizations) was higher with bevacizumab than with ranibizumab (24% vs. 19%, risk ratio 1.25; 95% confidence interval, 1.01 to 1.66). It is

important to note the limitations of the CATT[121] study in answering the questions many have regarding the safety of bevacizumab.

VEGF is an important molecule within the eye, which is not exclusively expressed by pathogenic neovascular tissues. Reduction of the number of anti-VEGF injections would lower the risk of potential disruption of normal physiologic processes mediated by VEGF, as well as possible ocular and systemic side effects.[122-124]

With regard to the current and future economic situation, savings in NHS expenditure are now an important government priority. The cost of drug treatment is an increasingly important consideration once equivalent efficacy and safety has been demonstrated. Ranibizumab costs significantly more than bevacizumab. [121] When this cost is extrapolated to the projected increased incidence of AMD with an ageing population this has important economic implications.

1.8 Tetracyclines

1.8.1 Anti-inflammatory actions of tetracyclines

Tetracyclines were first identified in 1948 as the natural fermentation product of the soil bacterium Streptomyces aureofaciens and six years later were chemically purified for the first time.[125] Since then, semisynthetic (SST) and chemically modified non-antibiotic tetracyclines (CMT) have been synthesized. The molecular modifications have served to enhance antibacterial activity,

improve tissue absorption and prolong their half life. Prolonged use of these compounds may lead to antibiotic resistance, however, chemical modification has been shown[126] to generate compounds devoid of antibiotic action therefore rendering potential for long term use in the treatment of complications related to oxidative stress.

Tetracyclines, are considered to possess clinically useful anti-inflammatory properties. Minocycline is a tetracycline derivative. In animals, tetracyclines have been shown to suppress delayed type hypersensitivity reactions, the rejection of transplants, [127] and the levels of immunoglobulins in the serum.[126]

There is increasing awareness of the role of oxidative stress in causing tissue damage and disease progression in a variety of conditions. A therapeutic role for the non- antimicrobial actions of the tetracyclines will be discussed in this context before considering their potential for treatment of AMD.

1.8.2 Therapeutic role for anti-oxidant actions of tetracyclines

Oxidative stress is a unifying mechanism for production of reactive oxygen species and plays a significant role in the manifestation of insulin resistance, atherogenic dyslipidaemia[129] and periodontal disease. [130] The term metabolic syndrome is used to describe a collection of risk factors for cardiovascular disease and type 2 diabetes. These include hyperglycaemia, hypertension, dyslipidaemia and a pro-inflammatory state often associated with obesity.[131]

Fluctuating glucose levels associated with higher levels of cytokines during hyperglycaemic spikes contribute to oxidative stress induced damage more so

than chronically elevated levels.[132] A nutritional overload resulting in excessive amounts of glucose and fatty acids leads to reactive oxygen species (ROS) production. Interactions between glucose and plasma proteins result in the formation of advanced glycation end products (AGE) which in turn initiate the production of TNF-α, IL-6, IL-18 and ROS contributing to chemical modification of lipoproteins and atherogenesis.[133] Patients with the metabolic syndrome show significant correlation with the prevalence of severe periodontal disease initiated by pathogenic bacterial plaque. Periodontal disease is characterised by destruction of tooth supporting soft and hard tissues. This is associated with high levels of inflammatory cytokines, other markers of inflammation and oxidative stress such as C-reactive protein and low density lipoproteins.[134] The link between cardiovascular and periodontal diseases is well documented while a causal relationship is more difficult to establish. Three months after intensive periodontal treatment in patients with a proven history of cardiovascular disease there was a significant reduction in levels of CRP and oxidised low density lipoprotein. [135]

Periodontal disease has a microbial aetiology and an inflammatory pathogenesis. Treatment strategy is based on controlling pathogenic bacteria to sub threshold levels for disease progression. This is achieved by performing periodontal root surface debridement. The use of adjunctive antimicrobials in the management of periodontal diseases is well established.[136] A systemic review comprising a meta analysis of 19 studies has shown that combining root surface debridement with sustained release antimicrobials was significantly

more effective in resolving periodontal disease than root surface debridement alone.[137] Minocycline and doxycyline gel were used as sustained delivery for placement in the periodontal pocket.

The tetracyclines are particularly versatile in their ability to combat oxidative stress, mop up free radicals and inhibit an excessive inflammatory response secondary to an antigenic stimulus.[137] Doxycycline hyclate has been shown to accelerate periodontal wound healing in diabetic mice[138] and humans.[139] Similar studies with Arestin (minocycline microspheres) have shown reduction in glycosylated haemoglobin measured by HbA1c levels and improved periodontal disease control compared to root debridement alone.[140] Adjunctive locally delivered doxycycline in periodontal pockets of smokers has been shown to be more effective than debridement alone[141] in reducing inflammatory periodontal disease. These studies suggest use of adjunctive tetracyclines have a beneficial effect on healing responses in the management of periodontal diseases in diabetic patients who smoke.

The tetracyclines have been shown to have cardioprotective effects related to suppression of inflammatory processes. Myocardial ischaemia is associated with the activation of matrix metalloproteinases and the serine proteinase plasmin. In a rat model, pretreatment with doxycycline has been shown to reduce myocardial infarct size by 37%.[142] Doxycycline inhibited plasmin while treatment with a broad spectrum matrix metalloproteinase inhibitor had no effect. This suggests that doxycycline may reduce myocyte death and contribute to cardioprotection. The antioxidant potency of minocycline has been

shown to be comparable with α-tocopherol, a synthetic form of vitamin E.[143] The radical scavenging actions of minocycline are consistent with its multi-substituted phenol ring similar to that of vitamin E belonging to the class of phenolic antioxidants. Minocycline was found to be more potent than α-tocopherol in its radical scavenging potency and inhibition of lipid peroxidation by 316 and 200 fold respectively. Minocycline hydrochloride has been shown to be very effective at quenching H_2O_2 levels. These actions of minocycline could explain its potency in reducing periodontal disease progression and cardioprotection in reducing the size of myocardial infarcts.[142]

A further study [144] investigated the effectiveness of Minocycline in attenuating oxidative stress induced neurotoxicity. The condition studied was vitiligo, an acquired and progressive disorder manifested by the selective destruction of melanocytes in the skin. A high level of hydrogen peroxide in plasma as well as within the skin lesions has been reported in vitiligo patients, which is thought to result in disappearance of melanocytes in this condition.

In the study, [144] to investigate whether minocycline rescues melanocytes from hydrogen peroxide induced apoptosis, cultured mouse melanocytes were treated with hydrogen peroxide in the presence or absence of minocycline. The results showed hydrogen peroxide decreased cell viability in a concentration dependent manner that was attenuated by Minocycline. The protective effect of Minocycline was associated with inhibition of c –Jun N terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK). The conclusions were that

minocycline may be used to prevent melanocyte loss in the early stages of vitiligo.

1.8.3 Minocycline as a neuroprotectant

It is well established [148] that inflammatory processes associated with neurodegenerative conditions and CNS trauma may exacerbate loss of neurones and enhance functional deficits. Before describing the proposed anti-inflammatory effects of minocycline a brief overview of the inflammatory response associated with spinal cord injury will be described.

A spinal cord injury triggers a series of secondary mechanisms which cause progressive damage to an increasing amount of tissue adjacent to the primary injury over a period of hours to weeks. Secondary mechanisms include vascular changes, haemorrhage, ischaemia, metabolic failure, free radical formation, lipid peroxidation, cytokine release and inflammation.[145, 146] These pathophysiological changes lead to necrotic together with apoptotic, neuronal and oligodendrocyte death, demyelination, cellular phagocytosis and axonal damage. [145, 146] The cellular reactions occur over hours to weeks, during which time therapeutic intervention may be achieved.

Early after spinal cord injury, microglia, the immunocompetent cells of the CNS, are activated and are the primary source of pro-inflammatory cytokines, namely tumour necrosis factor (TNF-α), interleukin-1beta (IL-1β), and IL-6.[147] This is followed by neutrophil invasion. Neutrophils produce and release many potential cytotoxic factors, including proteases, reactive oxygen species, and nitric oxide

and contribute to lipid peroxidation and protein nitration.[148] Strategies to prevent the detrimental effects of neutrophil invasion and toxicity are beneficial after spinal cord injury.[148]

Macrophages are the next immune cells to infiltrate the area. The activation of immune cells may be of benefit at some stages of spinal cord injury, other studies have shown that activated macrophages and associated cytotoxic products such as IL-6 contribute to secondary damage after spinal cord injury[148]

1.8.4 Mechanism of Minocycline's anti-inflammatory action in spinal cord injury

Minocycline has been suggested to exert its anti-inflammatory effects by modulating microglia, immune cell action and subsequent release of cytokines, lipid mediators of inflammation, matrix metalloproteinases (MMPs), and nitric oxide release. Caspases are a family of cysteine protease enzymes that play essential roles in apoptosis (programmed cell death), necrosis and inflammation. A common finding in many of the minocycline studies is the consistent reduction of caspase 1 mediated liberation of IL-1β.[149, 150] Similarly, minocycline suppressed TNF-α messenger RNA (mRNA) levels after spinal cord injury and prevented lipopolysaccharide (LPS) induced production of TNF-α in primary glial cultures.[151] In addition, the anti-inflammatory cytokine IL-10 was up regulated following minocycline application immediately after spinal cord injury.[152]

Therefore, modulation of pro-inflammatory cytokines may contribute to minocycline's neuroprotective effects. Minocycline may be neuroprotective by decreasing immune cell migration to sites of inflammation by altering levels of chemokines or chemokine receptor expression.

Minocycline can also modify lipid mediators of inflammation. Minocycline treatment inhibited both secreted and non secreted forms of phospholipase A2 in vitro.[153] In a rat model of focal ischaemia, pre-treatment with minocycline almost completely reduced protein (COX-2) levels and reduced prostaglandin (PGE2) levels by 55%.[149] Minocycline treatment has also been shown to abolish 5-lipoxygenase translocation to the nuclear membrane in PC12 cells subjected to oxygen-glucose deprivation.[154] PC12 is a cell line derived from rat adrenal medulla and is useful as a model for neuronal differentiation.[155] Hence lipid mediated inflammatory signalling may be attenuated at several steps following minocycline treatment.

An important role for MMPs in inflammation is well established. Both in vitro and in vivo studies have shown that minocycline treatment may reduce inflammation by inhibiting MMPs by diminishing cell infiltration and migration. Several MMPs especially MMP-12 increase after spinal cord injury and following brain haemorrhage and minocycline application downregulated MMP-12. [156]

Many studies[148] have shown that minocycline may be neuroprotective by regulating nitric oxide production. Microglia/macrophage release of nitric oxide is thought to mediate neurotoxicity in several neurodegenerative diseases. In

cultured microglial cells, minocycline reduced hypoxia and excitotoxin induced nitric oxide production.[157] In a gerbil model of global ischaemia, minocycline treatment reduced mRNA levels of cytokine derived nitric oxide synthase (iNOS) by 30% compared to saline treated controls, suggesting minocycline is effective at reducing iNOS expression in vivo.[158] In addition to an indirect effect on cell death through dampening inflammatory reactions, minocycline has been shown to have a direct effect on preventing apoptotic cell death.

1.8.5 Minocycline: An Anti-apoptotic agent

Apoptosis is an active and highly regulated form of cell death characterized morphologically by cytoplasmic shrinkage, chromatin condensation and fragmentation, membrane blebbing, and the disintegration of the cell into membrane bound intracellular inclusion bodies.[148] The apoptotic cell is subsequently removed by phagocytosis, a process, in contrast to necrosis, which does not induce an inflammatory response. Many of the features of apoptosis can be attributed to the specific cleavage of cellular proteins mediated by a family of cysteine proteases known as caspases. Mammalian caspases are synthesized as inactive pro-caspases, which require cleavage for activation and are often grouped according to function. Initiator caspases (caspase 2, 8, 9, and 10) function to activate effector caspases (caspase 3,6 and 7), which cleave several apoptotic substrates involved in the orderly breakdown of the cell.[159]

Two main apoptotic pathways, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway, have been described (Figure 9). The death receptor pathway involves binding of a death receptor (eg, Fas/CD95, TNF-α receptor) with its ligand and recruitment and activation of the initiator caspase-8, which in turn activate effector caspases (eg. caspase-3). The mitochondrial pathway involves activation of upstream pro-apoptotic factors that cause mitochondrial release of proteins cytochrome-c and second mitochondria derived activator of caspases (Smac), formation of an apoptosome, and sequestering of the inhibitor of apoptosis proteins (IAPs). These processes lead to the activation of initiator caspase-9, which in turn activates caspase-3. In addition, caspase independent cell death pathways involve the release of apoptosis inducing factor (AIF) from the mitochondria into the cytosol, where it translocates to the nucleus and contributes to DNA condensation and fragmentation. Once caspase-3 is activated, it cleaves many substrates including ICAD (inhibitor of caspase-activated deoxyribonuclease [CAD]) allowing for the liberation of CAD, an endonuclease that mediates the apoptotic internucleosomal cleavage of DNA.[148]

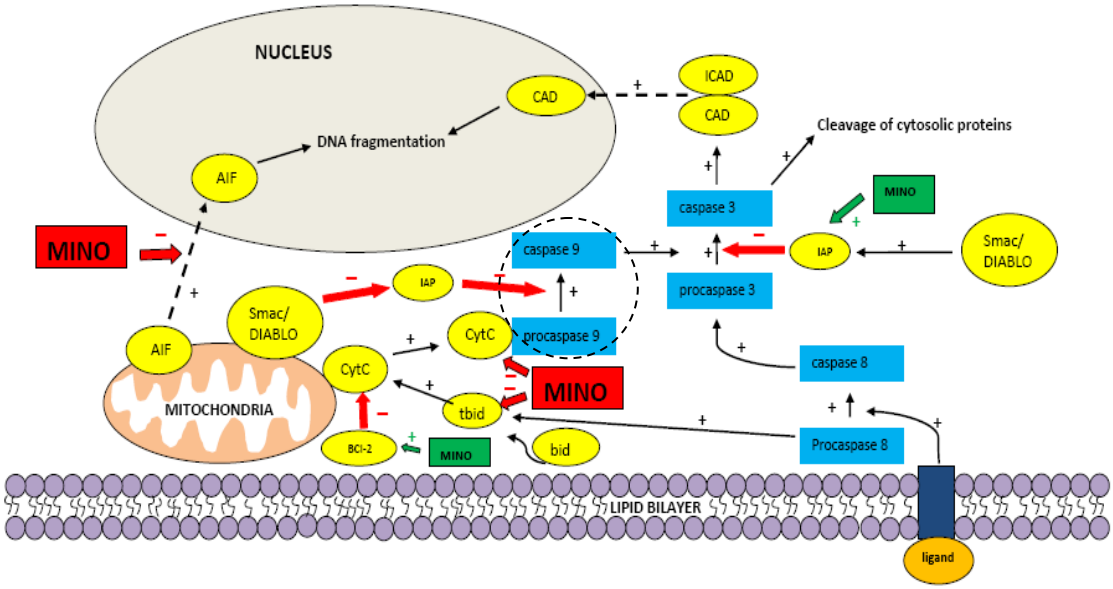

Figure 9: Apoptotic cell-death pathways and intervention following minocycline application. Minocycline may inhibit apoptosis by preventing the release of proapoptotic molecules (cytochrome *c* and apoptosis inducing factor [AIF]) (red signs = inhibition) and up-regulating antiapoptotic proteins including Bcl-2 and XIAP (green signs =stimulation). Dotted circle represents location of apoptosome.
(modified from Stirling DP, Koochesfahani KM, Steeves JD and Tetzlaff W. Minocycline as a neuroprotective agent. Neuroscientist 2005; 11:314)

The death receptor pathway involves activation of procaspase-8 and subsequent activation of effector caspases such as procaspase-3. The mitochondrial pathway involves cytochrome *c* release and formation of an apoptosome leading to activation of procaspase-9 and subsequent activation of procaspase- 3.

1.8.6 Mechanism of minocycline's survival promoting actions

Minocycline treatment reduced caspase-3 activation[160] following spinal cord injury (SCI). However, a direct effect of minocycline on caspase-1 and -3 activity

was ruled out using cell-free extracts, suggesting minocycline may act upstream of caspase activation.[161] In support of these findings, reconstitution experiments, by adding cytochrome c to isolated cytosol extracts of kidney cells, confirmed that minocycline acts at the level of the mitochondria, and not downstream of cytochrome c release.[162]

Minocycline mediated protection targets both caspase dependent (cytochrome c, Smac) and caspase independent (AIF) forms of cell death.[159] Minocycline may also prevent apoptosis by increasing levels of anti-apoptotic factors potentially upstream of cytochrome c release. In vitro studies have shown that minocycline treatment protected kidney epithelial cells against apoptosis induced by hypoxia, azide, cisplatin, and staurosporine by selectively increasing the anti-apoptotic protein Bcl-2 (both mRNA and protein).[162] Minocycline may also be neuroprotective by increasing levels of IAPs such as XIAP and thus preventing caspase activation and subsequent cell death. In support of this, it was demonstrated that in vivo pre-treatment with minocycline before ischemia/reperfusion injury of isolated rat hearts reduced both protein and mRNA expression of several initiator and effector caspases, diminished infarct volume, and lessened apoptotic cell death.[163] Concomitantly, minocycline treatment reduced caspase activity as well as cytosol levels of cytochrome c and Smac, and increased XIAP expression. In addition, treatment with minocycline alone without ischemia/ reperfusion also reduced caspase-1, 3, 7, 8, 9, and 12 expression below basal levels.[163]

Collectively, the results suggest that minocycline has a direct effect in inhibiting cell death by priming a “survival mode” rendering a cell less vulnerable to apoptotic stimuli. This may be due to up-regulation of anti-apoptotic proteins such as members of the Bcl-2 and/or IAP family that have been shown to antagonize the pro-apoptotic members of the Bcl-2 family and reduce caspase activation (Figure 9). In addition, minocycline may target the mitochondria directly and prevent the liberation of pro-apoptotic molecules such as cytochrome c, Smac, and AIF.

Minocycline’s anti-apoptotic combined with its anti-inflammatory properties protects cells from several death-inducing stimuli and likely explains minocycline’s neuroprotective effects in CNS trauma and disease.[148] Common signalling pathways may account for minocycline’s dual effects on cell death and inflammation, such as the p38 MAPK (mitogen activated protein kinase) pathway.[148]

1.8.7 Effects of minocycline in the eye

Several clinically diverse eye conditions involve inflammation as an important factor in their aetiology. The meibomian glands are modified sebaceous glands present in the eyelids that produce the lipid component of the tear film[6] and chronic inflammation of the glands produces a condition known as blepharitis.[6] Minocycline has been used to treat meibomian gland dysfunction.[164] In a prospective study of sixteen patients, to evaluate clinical impact, tear related parameters and meibomian gland dysfunction, three months of oral minocycline

resulted in clinical improvement in all cases of meibomianitis. Minocycline has also been shown to have neuroprotective properties in retinal ganglion cells.[165] To investigate the effect of minocycline on the survival of retinal ganglion cells, a rat model of myelin oligodendrocyte glycoprotein induced experimental encephalomyelitis, an animal model of brain inflammation, was used. Inflammation of the optic nerve, known as optic neuritis in this model was diagnosed by recording visual evoked potentials. Visually evoked potentials record the electrical activity of the brain with a visual stimulus. The study[165] demonstrated minocycline induced neuroprotection is related to direct antagonism of multiple mechanisms leading to neuronal cell death such as induction of anti-apoptotic intracellular signalling pathways and a decrease in glutamate excitotoxicity. The conclusion was that minocycline exerts neuroprotective effects independent of its anti-inflammatory properties.

Recently further research[166] has been undertaken to elucidate the role of nuclear factor kappa B and mitogen activated protein kinases in light induced apoptosis of photoreceptors in culture and to explore the potential inhibitory effect of minocycline and sulforaphane on apoptosis. Mitogen-activated protein kinases (MAPKs) are serine or threonine kinases that play an important role in signal transduction from the cell surface to the nucleus. The mammalian MAPKs include p38, an extracellular signal-regulated kinase (p44/42), and Jun N-terminal kinase (JNK). It has been shown that a balance between the survival-promoting p44/42 pathway and the death-promoting the p38 and JNK pathways determine the fate of cells. Nuclear factor-κB (NF-κB) is a ubiquitous

transcriptional factor that regulates a broad range of genes and plays a role in cell death or survival. The MAPK and NF-κB pathways may have both positive and negative effects on apoptosis, depending on the types of cells and stimuli.

Researchers [166] investigated the expression of immunologic signalling molecules in light-induced apoptotic mouse 661W cells in culture. Cellular pathways regulating the light-induced photoreceptor apoptosis process, and the potential therapeutic effect of minocycline and sulforaphane on light-induced photoreceptor apoptosis was also studied.[166] Sulforaphane is a naturally occurring isothiocyanate found in broccoli. The 661W photoreceptor cell line was originally isolated from transgenic mice. However, when compared with the in vivo photoreceptor cells, the 661W cells have limitations, they proliferate in culture, unlike photoreceptor cells in the differentiated retinas, they do not exhibit cone photoreceptor morphology, such as formation of outer-segment–like membranes, they do not express outer segment structural proteins, such as peripherin/rds and ROM1, and they do not express RPE65, an important determinant in rhodopsin regeneration as part of the visual cycle and light-damage susceptibility in mice.[166]

During the study, [166] 661W cells in culture were exposed to light for four hours and were used as a model to investigate photoreceptor apoptosis. The results showed pretreatment of 661W cells with minocycline inhibited light-induced photoreceptor apoptosis and the down-modulation of the NF-κB p65 subunit. However, minocycline had no direct effect on the expression of the MAPKs. Minocycline inhibited proteolytic cleavage of NF-κB proteins. These

observations suggest that minocycline inhibits light-induced photoreceptor apoptosis partly through an NF-κB-dependent mechanism.

Results[166] showed pre-treatment of 661W cells with sulforaphane inhibited light-induced photoreceptor apoptosis and inhibited down-modulation of the p65 subunit of NF-κB, but it had no effect on the expression of the MAPKs. Like minocycline, sulforaphane also inhibited the proteolytic cleavage of NF-κB proteins. The study[166] suggests that sulforaphane inhibits light-induced photoreceptor apoptosis partly through an NF-κB-dependent mechanism.

1.9 Experimental Models for AMD research

With the use of specific measures, RPE cells in vitro can be made to differentiate into a phenotype resembling RPE in vivo, displaying polarity, a columnar morphology, the presence of gap or even tight junctions and the presence of microvilli at their apical surface.[167, 168]

RPE cells may be cultured on filter systems, to allow evaluation of polarized growth factor secretion or in co-culture systems with choriocapillaris endothelial cells or neuroretinal explants.[167] Exposing the cells to substances such as rod outer segments, or advanced glycation end products (AGE), provides ways to investigate mechanisms involved in AMD, such as lipofuscin formation and oxidative stress.[168] Increased protein cross linking and the accumulation of AGEs develop in ageing human Bruch's membrane.[169] In addition to effects on

the structural integrity of Bruch's membrane, AGEs also exert pathogenic effects by interaction with cell receptors, the best characterised is RAGE (receptor for AGEs). RAGE has been implicated in long term diabetic complications, neurodegenerative diseases, acute and chronic inflammatory disorders and cancer.[170] RAGE is a multi-ligand receptor and in addition to binding AGEs, RAGE also acts as a signal transduction receptor for amyloid β (Aβ) and S100/calgranulins. The S100/ calgranulins are a group of proteins involved in inflammatory responses. Amyloids are proteinaceous deposits contributing to the pathology of several pathological conditions. Amyloid is a component of drusen, with deposition thought to be AMD specific and several studies have addressed the molecular contribution of amyloid to the development of AMD.[171, 172]

Ma et al (2007) [173] investigated the hypothesis that activation of RAGE by its ligands including AGEs, amyloid-β peptide (Aβ), and S100B/calgranulins modulates secretion of VEGF by retinal pigment epithelial (RPE) cells. The ARPE-19 cell line was used and VEGF secretion and gene expression were assessed by ELISA and quantative real time PCR. The results[173] demonstrated ARPE-19 cells basally secreted VEGF under normal cell culture conditions. Immobilized ligands of RAGE increased VEGF secretion in a dependent manner. RAGE mediated upregulation of VEGF secretion by ARPE-19 cells was largely dependent on NF-κB. Inhibition of NF-κB effectively abrogated the response of RPE cells to RAGE ligands under conditions that would normally upregulate VEGF secretion. The authors[173] used parthenolide, an active

substance in the medical herb Feverfew (Tanacetum parthenium) traditionally used in the treatment of inflammation. The authors[173] concluded that the NF-κB dependent nature of RAGE stimulated VEGF secretion in RPE cells renders this pathway together with the RAGE axis itself a potential therapeutic target for the reduction of neovascular stimulus in eyes at high risk for progression of macular degeneration and further study would be necessary to define the role of RAGE and its ligands in the progression of AMD. [173]

Howes et al (2004)[174] evaluated RAGE mediated cellular activation in the aetiology of human retinal aging and disease. Maculas of human donor retinas from normal eyes and eyes with early age related macular degeneration and advanced macular degeneration were assayed for AGE and RAGE by immunocytochemistry. Cultured ARPE-19 cells were challenged with known ligands for RAGE, AGE and S100B to test for activation capacity. Immunocytochemistry, real time polymerase chain reaction (PCR), immunoblot analysis and the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay were used to determine the consequences of RPE cellular activation.[174] The results[174] demonstrated little immunolabelling for AGE or RAGE in photoreceptor and RPE cell layers in normal retinas. When small drusen were present AGE and RAGE were identified in the RPE or both RPE and photoreceptors.[174] In early and advanced AMD the RPE and remnant photoreceptor cells showed intense AGE and RAGE immunolabelling. Both AGE and S100B activated cultured RPE cells, as revealed by upregulated expression of RAGE, NF-κB nuclear translocation and apoptotic cell death.[174]

Similarly, an in vitro RPE system that was challenged with matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPS) and TNF-α was developed to study the pathogenesis of sub-RPE deposit formation.[175] ARPE-19 cells treated with TNF-α or MMP-2 showed a reduction in all types of sub-RPE deposit and TNF-α stimulated MMP-9 production.

1.9.1 Response of RPE cells to Oxidative stress

Continual phagocytosis of photoreceptor segments and the high blood flow through the choriocapillaris results in oxidative stress via the production of hydrogen peroxide.[17]

The RPE cells are responsible for the maintenance of the blood retinal barrier that is dependent on the function of tight junctions between the cells. Breakdown of this barrier may allow immune cells to gain access to the retina with subsequent release of pro-inflammatory cytokines. Tight junction proteins ZO-1 and occludin are present in ARPE-19 cell lines.[61] Adherens junctions maintain the morphology of the RPE cells and various factors may disrupt the function of both tight and adherens junction proteins. Treatment of RPE cells with hepatocyte growth factor results in redistribution and loss of protein components.[176]

The effect of oxidative stress on the ARPE-19 cell line, junctional integrity and the induction of stress proteins has been studied.[177] Results showed ARPE-19 cells response to oxidative stress induced with hydrogen peroxide differed with time in culture. [177] The cells cultured for five weeks were less sensitive to

oxidative stress than those cultured for one week. The more differentiated cells expressed increased levels of heat shock proteins, Hsp27 and Hsp70 and it was suggested this may play a protective role by increasing intracellular glutathione, and also conferring resistance to apoptosis.[177]

1.9.2 Response of RPE cells to effects of T lymphocyte products

T lymphocytes are integral to the initiation, maintenance and resolution of the immune response. Human retinal epithelial cells (HRPE) express cell adhesion molecules and chemokines for leukocytes. [178,179] The T cell receptor –CD3 complex and associated cell surface receptors are key mediators of lymphocyte activation when antigen is presented to lymphocytes by antigen presenting cells bearing HLA-DR antigens.[180] Interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) are two chemokines responsible for the majority of pro-inflammatory cytokine induced human RPE derived leukocyte chemotactic activity.[178] IL-8 is chemotactic for neutrophils, lymphocytes and eosinophils whereas MCP-1 is chemotactic for monocytes and lymphocytes. Interferon-γ (IF- γ), a lymphokine produced by T-lymphocytes has been shown to have important immunomodulatory effects on human RPE cells including the induction of human leukocyte antigen (HLA)-DR and intercellular adhesion molecule.[178-180] Research was conducted to examine the effects of T-lymphocyte products on human retinal pigment epithelial (HRPE) cell interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) secretion and gene expression.[178] Results of this study showed T lymphocyte secretions induce HRPE IL-8 and MCP-1 gene expression and secretion. TNF and IF-γ

appear to be necessary components of T-lymphocyte conditioned media for the induction of HRPE IL-8 and MCP-1. IL-2 did not appear to modulate cytokine induced HRPE IL-8 or MCP-1. The study[178] suggested lymphokine secretions containing TNF-α and lF-γ may affect leukocyte recruitment at the blood retina barrier by inducing local chemotactic cytokines IL-8 and MCP-1 from the vascular endothelium and HRPE. Expression of HLA-DR and intercellular adhesion molecule-1 on HRPE cells and vascular endothelium was also induced, resulting in leukocyte accumulation, activation, and trafficking at the retinal-choroidal interface.

1.9.3 Glycated albumin

Various stimuli, such as IL-1β, tumour necrosis factor (TNF-α), and glycated human serum albumin (GHSA) have been shown to stimulate human RPE IL-8 and MCP-1 secretion.[181-183] GHSA is a glycation adduct. Protein glycation occurs in both normal and hyperglycaemic serum when glucose nonenzymatically attaches to lysine residues of the proteins and forms labile Schiff base intermediates that undergo Amadori rearrangement and lead to the relatively stable early adducts ketoamine or fructosamine.[184] The ambient glucose concentration and protein turnover rate determines whether the early glycated proteins may eventually form irreversible advanced glycation end products (AGEs). Glycated proteins increase under hyperglycaemic conditions. Plasma levels of GHSA may vary from normal (400µg/ml) to 1000µg/ml in diabetics. [185]

Enhanced protein glycation can be observed in experimental diabetes mellitus. A close correlation has been demonstrated between plasma glucose concentration and the degree of albumin glycation.[186] Therefore, the amount of glycated albumin has been used as an index of short to intermediate term glycaemic control. [186] Increasing evidence has suggested that early glycated albumin is not just an index of glycaemia or the precursor of AGEs. Glycated albumin itself, may have important direct effects on cellular function and hence play a pathophysiological role in microvascular complications of diabetic nephropathy and retinopathy. [181,187-190] Further evidence for the pathophysiological role of early glycated albumin is suggested by the existence of specific receptors for early glycated albumin.[191-195] These receptors differentially bind Amadori modified glycated albumin, but not AGEs, suggesting that the functional role of early glycated albumin may differ from AGEs. An example of this is that early and advanced glycation products differently affect retinal microvascular cell growth.[189]

1.9.4 Hypoxia

In conditions of hypoxia, although oxygen is present demand exceeds supply. Aerobic organisms are dependent on the efficient oxygen dependent release of energy, stored in the chemical bonds of glucose through oxidative phosphorylation. Hypoxic conditions are not wholly pathological as uptake of oxygen is restricted in many species such as whales and burrowing animals.[196] Many indigenous human populations live at high altitudes so experiencing lowered oxygen tension and relative hypoxia. Acute hypoxia may be caused by

decreased blood flow, vessel contraction or occlusion and inflammatory conditions. Chronic hypoxia may be caused by high altitude. Specialised tissue such as the carotid body is sensitive to lowered oxygen tension. Hypoxia mediates effects on the nervous, respiratory and cardiac systems.[196] Reduced oxygen tension in the liver and kidney results in the production and release of erythropoietin (EPO) with subsequent increase in the production of red blood cells.[196]

The retina is the most metabolically active tissue in the human body, and hence the retina is highly sensitive to reduction in oxygen tension.[196]Therefore, any disturbances in oxygen delivery into the retina as a result of lung or cardiac disease, or local occlusive vascular diseases with inflammation in the eye will ultimately lead to hypoxia conditions in the retina that may elicit development of AMD. [197, 198]

1.94.1 Regulation of vascular endothelial growth factor

In adults VEGF has been widely considered to be a pathological factor in the development of choroidal neovascularisation (CNV), However, VEGF has important functions in the adult healthy retina. VEGF protects the retinal pigment epithelium (RPE), Muller cells, photoreceptors and retinal neurons. [199, 200]

VEGF expression and secretion are regulated on many levels by various factors, such as different transcription factors, [201] protein kinases [202] and receptor signalling. [203]

For ocular tissue, differential involvement of mitogen activated protein kinases (MAPK) has been shown,[204] as p38 is involved in constitutive VEGF expression and secretion, while extracellular signal regulated kinase 1/2 accounts only for oxidative stress induced VEGF increase, which is a likely transient phenomenon.[205]

Klettner et al (2013)[206] investigated the regulation of constitutive vascular endothelial growth factor in porcine RPE/choroid organ cultures. [206] VEGF content was evaluated with ELISA. The influence of several molecular factors was assessed with commercially available inhibitors. For toxicity measurements of inhibitors, primary RPE cells of porcine origin were used and toxicity was evaluated with methyl thiazolyl tetrazolium assay. The results showed VEGF secretion as measured in RPE/choroid organ culture was diminished after long term (48 hour) inhibition of VEGF receptor 2 by VEGF receptor 2 antagonist SU1498. VEGF secretion was also diminished by after phosphatidylinositol 3 kinase was inhibited by Ly294002 for 48 hours. Coapplication of substances did not show an additive effect, suggesting that they use the same pathway in an autocrine positive VEGF regulation loop.

Biochemical inhibitors were used, as small interfering RNA or specific antibodies were not feasible or affordable. Biochemical inhibitors have been well described in the literature. [207-209] The specificity of some biochemical inhibitors is under debate [210] which is a limitation of this study.

In endothelial cells VEGF induces the expression of HIF-1α, an important regulator of VEGF in hypoxia.[211] HIF-1α induced upregulation of VEGF in hypoxia has been shown for RPE, [201] however, in the study of Klettner et al [206] HIF-1α did not have an effect on constitutive VEGF secretion.

Klettner et al [206] reported that inhibition of the transcription factor NF-κB by biochemical inhibitors exhibited a strong effect on VEGF secretion at all time points tested, 6 hours, 24 hours and 48 hours. This suggested constant influence of NF-κB on constitutive VEGF secretion. NF-κB is an important regulator of the innate immune response, [212] inhibitor of autophagic processes[213] and involved in ageing. [214] All these factors are associated with development of AMD. [215] Control of VEGF expression regulated by NF-κB may be an interesting target for prevention of AMD development.

Klettner et al [206] also showed that p38 inhibition for 24 and 48 hours regulated constitutive VEGF expression and secretion. The pattern of VEGF reduction in p38 inhibition resembled the pattern displayed by NF-κB inhibition. The effects of p38 and NF-κB inhibition appeared to be additive, suggesting the involvement of different pathways. VEGF secretion was completely abolished by coapplication of p38 and NF-κB inhibitors. [206] Similar inhibition of VEGF secretion is usually only seen when extracellular VEGF inhibitors such as bevacizumab or ranibizumab are used.[216] This suggests that these pathways may offer alternatives for VEGF inhibition and possible therapeutic opportunities to delicately alter the amount of available VEGF in the retina. However, the results of Klettner et al [206] should be interpreted in view of the limitations of their

study. There are limitations in using biochemical inhibitors, and an in vitro porcine model may not reflect functioning in the aged human eye.

1.94.2 Measurement of retinal hypoxia

Due to the small calibre of retinal vessels, it is not possible clinically to directly measure blood flow, perfusion pressure or oxygen tension of the retina and optic nerve. Therefore, other indirect non invasive methods to measure these parameters are used. Photography, spectral or pulsatile changes of vessels, confocal scanning laser Doppler flowmeter, colour Doppler imaging, laser Doppler flowmeter, and Heidelberg retina flowmeter are examples of techniques that have been used to assess the blood flow of retina and optic nerve head.[217]

1.94.3 Hypoxia in AMD

It has been suggested that foveolar choroidal blood flow decreases in patients with AMD.[218] As such, the reduction in choroidal blood flow could lead to ischaemia and hypoxia resulting in a plausible role for hypoxia in the development of CNV. This hypothesis is supported by deposition of materials and thickening of the RPE-Bruch's membrane complex that occurs in AMD. This process may impede the diffusion of substances [219] and would increase the distance that oxygen must travel from the choriocapillaris to the photoreceptors, further reducing the availability of oxygen to the outer retina. [220]

Studies in patients with asymmetric AMD disease suggest that ocular circulatory abnormalities may have role in the development of CNV. [221, 222]

Grunwald et al[197] used laser Doppler flowmetry to assess relative foveolar choroidal blood velocity, volume, and flow. The study included a control group and the AMD study eyes were subdivided into three groups according to increasing risk for AMD. The results demonstrated that choroidal circulatory parameters decreased with an increase in severity of AMD features associated with risk for the development of CNV. This suggests a role for ischaemia in the development of AMD.

Metelitsina TI et al [198] reported the results of a prospective study in a cohort of patients with AMD at risk of development of CNV. The purpose of the study was to investigate in a longitudinal fashion whether fovelolar choroidal blood flow decreases before the development of CNV and whether changes in blood flow precede the formation of CNV. The study was conducted over a time period of 5 years.

All study eyes[198] had typical ophthalmoscopic features of AMD at baseline but no signs of CNV. CNV developed in 21% of eyes during the study. In eyes with CNV, foveolar choroidal circulatory measurements decreased by approximately 10% before the formation of CNV. This contrasted with findings in eyes that did not go on to develop CNV where choroidal circulatory parameters increased. Eyes with lower baseline foveolar choroidal flow were more likely to show visual loss than eyes with higher baseline parameters. These results[198] suggest that

decreases in foveolar choroidal circulation precede the development of CNV in AMD and may play some role in its development.

Alteration of transepithelial transport may function in the pathogenesis of age related macular degeneration. In 20-25% of patients with AMD, drusen become large and confluent [223] and establish large diffusion barriers between blood vessels and the RPE. This may lead to development of areas with reduced supply of oxygen and glucose, mimicking hypoxia. [17, 224] These regions of hypoxic stress may cause degeneration of adjacent photoreceptors and subsequent loss of vision in areas of drusen. A further consequence of metabolic stress might be the induction of choroidal neovascularisation (CNV), the most severe complication in the aetiology of age related macular degeneration.[223] Hypoxia reduces the secretion of pigment epithelium derived growth factor (PEDF) by the RPE. [225] PEDF is a potent antiangiogenic factor. The hypoxic theory is supported by the observation that choroidal neovascular membranes contain high amounts of advanced glycation endproducts that are generated by reactive oxygen species during reduced metabolism. [226]

Recently, automatic retinal oximetry, which measures the haemoglobin oxygen saturation in retinal vessels has become a promising indirect method to quantify hypoxia in the retina. [227] Newer techniques confirmed previous hypotheses that because of decreased choroidal circulation, hypoxia is an important factor affecting the progression of AMD. [17,197,198,228]

1.94.4 HIF gene

Transcriptional regulation by an oxygen dependent transcription factor, hypoxia inducible factor (HIF) was discovered in the 1990s. HIF was originally found and characterised from human hepatoma cell culture where it was found to influence transcription of the erythropoietin gene. [229]

Specifically, HIF is a heterodimeric transcription factor composed of a labile oxygen sensitive alpha subunit (120kDa) and a stable constitutively expressed beta unit (92kDa). They bind to DNA at specific locations termed hypoxia responsive elements (HREs).[230]

Human choroidal neovascular membranes associated with AMD contain HIF.[231] Since the production of VEGF in human retinal pigment epithelium cell culture is regulated by HIF[232] any disturbance in HIF and associated inhibitory protein function instigated by oxidative stress and inflammation is likely to affect the formation of choroidal neovascularisation in the eye.

Cells exposed to hypoxic stress either manifest an adaptive response or undergo cell death. The ability of cells to maintain a balance between adaptation and cell death is regulated through several mechanisms through which cells strive to prevent stress induced cellular damage. [232]

Oxidative stress has been implicated in the senescence of RPE cells and the pathogenesis of AMD. It occurs when the level of reactive oxygen species (ROS) exceeds the detoxifying capacity of antioxidants or molecular chaperones. [233] ROS which are formed damage lysosomal membranes of RPE

cells. This weakens their ability to remove the constant stream of metabolites released from rods and cones. In senescent RPE cells, this usually results in the accumulation of lipofuscin in lysosomes. [234] ROS and HIF are involved in stimulating angiogenesis in the retina.[235] Intermittent hypoxia, followed by reoxygenation have been shown to determine the production of ROS, so leading to HIF activation and accelerated ageing and to the appearance of age related diseases. [236] Molecular responses ultimately lead to increased expression of VEGF which is the major growth factor triggering neovascularisation in the exudative form of AMD.

Chapter 2

2.0 Rationale

The following section provides an explanation for the rationale of the pre-clinical and clinical study presented in this thesis.

2.1 Introduction

Neovascular AMD is the leading cause of blindness in people ≥50 years of age in the Western world. The condition is characterized by choroidal vascularisation that has a complex pathogenesis involving neovascular growth, vascular leakage, matrix deposition, remodelling and inflammation. At present, the most effective treatment consists of VEGF blockade necessitating repeated intravitreal injections and a prolonged period of intensive follow up for patients.

2.2 Retinal pigment cell physiology

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells forming the outer blood retinal barrier.[237] As a layer of pigmented cells the RPE absorbs the light energy focused by the lens on the retina. The RPE transports ions, water and metabolic end products from the subretinal space to the blood. The RPE takes up nutrients such as glucose, retinol, and fatty acids from the blood and delivers these nutrients to photoreceptors. A well recognised process known as phototransduction occurs when absorption of light is converted to an

electrochemical signal. This process involves a substance, retinal, that is constantly exchanged between the photoreceptors and the RPE. This is known as the visual cycle.[237] The photoreceptors contain high amounts of photosensitive molecules and are exposed to intense levels of light. This leads to accumulation of photo-damaged proteins and lipids. In addition, the retina itself generates photo-oxidative radicals. During each day, the concentration of light induced toxic substances increases inside the photoreceptors.[238] Light transduction by photoreceptors is dependent on the proper function and structure of proteins and membranes. Therefore, to maintain the excitability of the photoreceptors, the photoreceptor outer segments (POS) undergo a constant renewal process. The tips of the POS that contain the highest concentration of radicals, photo-damaged proteins, and lipids are shed from the photoreceptors. Through co-ordinated POS tip shedding and formation of new POS, a constant length of POS is maintained. Shed POS are phagocytosed by the RPE. The process of POS shedding and phagocytosis is under circadian control. [240] An increase in oxidative stress due to a reduction in protective mechanisms or an increase in number and concentration of active photo-oxidative reactive species are believed to contribute to the pathogenesis of AMD.[240]

2.3 Inflammatory pathophysiology of AMD

It has been postulated that a starting point for the pathological process of AMD is the accumulation of lipofuscin in the RPE. [241] The onset of this chain of events is based on age dependent changes of the RPE. This includes a reduction in

the cell density of the RPE cells which may itself result from apoptosis, which is caused by accumulation of toxic substances. The increase in the amount of reactive oxygen species destabilizes intracellular membrane compartments such as lysosomes and mitochondria. The resulting decrease in metabolic efficiency produces more lipofuscin and reactive oxygen species. This generates a pro-inflammatory state promoting the development of AMD. Cytokine production including vascular endothelial growth factor release results in pathological neovascularisation leading to neovascular AMD.[241] The inflammatory process and cytokine release involves complex pathways culminating in the pathological end point of neovascularisation. There is therefore a scientific rationale for targeting the inflammatory processes prior to the development of neovascularisation. The advantage of such an approach would be to implement therapy at an earlier stage of the pathological process so avoiding repeated anti VEGF injections with no defined end point to treatment. Repeated anti VEGF therapy imposes an increasing burden both for individual patients as well as the healthcare system.

2.4 Rationale for anti-inflammatory therapy in neovascular AMD

Anti-inflammatory agents such as intravitreal steroids are well established therapeutic modalities for ocular inflammatory conditions such as uveitis. However, their prolonged use is widely recognised to be associated with raised intraocular pressure and cataract formation. Minocycline has been recognised to have anti-inflammatory, antiapoptotic and neuroprotectant functions. It has also been shown to protect photoreceptors from light and oxidative stress.

However, the use of minocycline as a therapeutic agent in age related macular degeneration is yet to be established.

The preclinical part of this study investigates the effects of minocycline on RPE cells in culture and the effects of minocycline on MCP-1 and IL-8 cytokine release.

2.5 Rationale for clinical study

Photodynamic therapy (PDT) was the first treatment shown to be of clinical benefit for most patients with subfoveal neovascular AMD. Standard photodynamic therapy is a two step process involving an initial intravenous infusion of verteporfin followed by irradiance with a 689nm laser for 83 seconds beginning 5 minutes after the infusion delivering a total energy of 50J/cm^2. Verteporfin binds to low density lipoprotein receptors in the plasma during the infusion, which are then prefentially bound by choroidal neovascular tissue which then expresses low density lipoprotein receptors. Irradiation of the neovascular lesion by the laser creates toxic oxygen species that induce thrombosis and closure of the choroidal neovascularisation.

Although the thrombosis predominantly affects choroidal neovascularisation, there is evidence of damage both to the choriocapillaris and retinal pigment epithelium. [242] The Verteporfin in Minimally Classic CNV Study examined the effects of both reduced fluence and standard fluence in minimally classic lesions. In such lesions, the study suggested that reduced fluence may be beneficial.[243] Reduced- fluence PDT with 25 J/cm^2 reduces choroidal perfusion,

inflammation, vascular leakage and VEGF upregulation that is associated with standard fluence PDT. [244] Reduced fluence PDT was used only once during the clinical study presented in this thesis.

Anti-inflamatory agents such as intravitreal triamcinolone have been used as an adjunct for PDT to limit further VEGF upregulation initiated by the therapy.[245]

Combination therapy has been shown to be beneficial when compared with PDT monotherapy in terms of functional results. [244,245] Triamcinolone has been associated with an increased risk of cataract formation [246] and raised intra-ocular pressure.[247] It was not until recently that replacement of triamcinolone with dexamethasone was considered.[248] Triamcinolone that is injected as a suspension has prolonged effects, particularly with regard to raised intra-ocular pressure. Dexamethasone which is injected as a solution is more rapidly cleared from the vitreous; as there is no sustained release due to suspension, there is reduced risk of steroid induced side effects. [248]

Dexamethasone has anti-inflammatory, antifibrotic and anti-VEGF effects. [249-251] Further, its antifibrotic effects are reduced in the presence of VEGF [252] so that the combination of dexamethasone with anti-VEGF therapy may assist with dexamethasone's antifibrotic effects. Dexamethasone may reduce endothelial dysfunction and inhibit VEGF induced vascular dysfunction.[249]

At the molecular level, dexamethasone exerts its anti-inflammatory effect by interfering with the activation of pro-inflammatory genes without affecting factors that inhibit inflammation.[253]

Minocycline is a semisynthetic derivative of tetracycline with a longer half life and improved penetration through the blood-brain barrier. Minocycline has been shown to protect melanocytes from apoptosis induced oxidative stress in vitiligo, a progressive disorder manifested by the selective destruction of melanocytes in the skin. [254] Leung et al [255] investigated whether minocycline and its structurally related analogues would protect photoreceptor cells in primary bovine culture from light and oxidative stress. Minocycline was shown to protect photoreceptors in culture but within a narrow therapeutic range of concentrations. [255]

It was postulated that minocycline may act as an adjunct for neovascular AMD in the combined treatment regimen used in the clinical study presented in this thesis. A reduced dose of ranibizumab (0.3mg in 0.05ml) was used in this study given the combined angio-occlusive effect of PDT and the effects of dexamethasone and minocycline.

Combination therapy in exudative AMD is aimed at targeting the different pathways in the disease process while reducing or counteracting the adverse effects of individual therapeutic agents. The adverse ocular effects of PDT include choroidal hypoperfusion, inflammation, upregulation of VEGF production and scarring induced by irradiance. The adverse ocular effects of ranibizumab include the potential detrimental effects of chronic VEGF blockage on the function of the photoreceptors. Adverse ocular effects of dexamethasone include raised intra-ocular pressure and cataract. Minocycline as an individual agent has no specific adverse ocular effects except the rarely reported

association with idiopathic intracranial hypertension. [248] In terms of safety, potential negative synergistic effects of minocycline and PDT exist. In addition, a negative synergistic effect of inducing ischaemia in the choroidal and retinal circulation followed by atrophy exists with combined PDT, intravitreal ranibizumab and dexamethasone.

In the clinical part of this study the safety and effectiveness of the combined therapy of intravitreal ranibizumab and dexamathasone, oral minocycline and verteporfin photodynamic therapy for subfoveal choroidal neovascularisation (CNV) secondary to age related macular degeneration (AMD) was assessed. The cause of AMD is not known, but it is known that many factors contribute to the final pathological end point of abnormal blood vessels. These factors involve cytokines and complex molecular reactions with a delicate balance between stimulatory and inhibitory factors. Combined therapy, using agents that act at different points in the pathological pathway and therefore synergistically have the potential to be more efficiaous than monotherapy. This concept is gaining ground and many clinical trials have incorporated a number of combination modalities.

2.6 Hypothesis and Aims

Ischaemia and the resulting hypoxia at a cellular level are considered important aetiological factors in the pathogenesis of AMD the evidence for which has been discussed in Chapter 1 above.The biochemical changes of ageing lead to an accumulation of toxic substances within the RPE cell and in combination with

hypoxia are hypothesised to promote the process of inflammation. Inflammation and the release of cytokines are hypothesised to instigate pathological neovascularisation leading to neovascular AMD.

Oxidative stress and inflammation are considered to be significant factors in the progression of metabolic conditions and minocyline has been shown to have therapeutic potential in inflammatory conditions and as a neuroprotectant as has been discussed in section 1.8.3 above. In this study, it is hypothesized that minocycline has a therapeutic role for the inflammatory component of neovascular AMD.

The aim of this study is to investigate the mechanisms of action of minocycline on retinal pigment epithelium (RPE) cells in culture and to discuss the findings in the context of clinical trial data from patients with age related macular degeneration (AMD) receiving minocycline treatment. Minocycline is proposed to act as an anti-inflammatory agent and mediate its effects through reduction of oxidative stress and inhibition of apoptosis. The aims of this study will be achieved by culture of ARPE-19 cells using glycated human serum albumin and hypoxia as a source of oxidative stress.

The effects of hypoxia and glycated human serum albumin on RPE cell viability and apoptosis and expression of pro-inflammatory cytokines, MCP-1 and IL-8 will be investigated. The effects of minocycline on apoptosis rates and MCP-1 and IL-8 production will be studied.

Chapter 3

3.0 Pre-clinical study

3.1 Introduction

Primary cultures have been commonly utilized for RPE research as it is common for differentiated cells to lose their specialised properties after multiple passages. Primary cultures of RPE cells from various species have been shown to retain many normal physiological functions, including the ability to transport retinoids [238] and phagocytose rod outer segments. [239, 240]

Human eyes for use in primary culture are often difficult to obtain and as such cultures usually require purification before a uniform population of cells is established. Primary cultures may also exhibit physiological differences caused by donor to donor variability.

3.2 ARPE-19 cell line

ARPE-19 is a spontaneously arising human RPE cell line that has become a good alternative, though not superior to primary culture.[256]

Dunn et al [257] extensively characterised this cell line in 1996 and demonstrated several phenotypic characteristics similar to those of primary RPE. Available passage numbers for this cell line are limited to those above p20.

The transcriptional profile of RPE cultures including ARPE-19 cells and its proximity to native RPE has been described.[258] Culture conditions were varied

and genes expressed were examined with array analysis. Hierarchical cluster analysis showed that confluent and differentiated serum withdrawn cultures clustered closest to native RPE, and that serum segregated cultured cells from native RPE. This suggests that serum influences multiple cellular functions including protein processing, signal transduction, cell cycle, metabolism, and transcription. As the outer blood retinal barrier is composed of the RPE and Bruch's membrane, the RPE is probably partially shielded from serum by Bruch's membrane. Hence RPE cells in vivo are not significantly exposed to serum.

3.3 Materials and Methods

3.3.1 Cell Culture

The ARPE-19 cell line (American type culture collection, ATTC) were utilised in this study. Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing glutamax supplemented with 10% foetal bovine serum (GIBCO, Invitrogen, UK) and 1% penicillin-streptomycin (1000 units of penicillin/ 1mg streptomycin, GIBCO, Invitrogen, UK). Cells were incubated in 5% CO_2 at 37°C.

3.3.2 Cell passage and maintenance

Cells were cultured in T75 flasks (Nunc, Thermo-fisher scientific, UK). A volume of 13ml of media was used per flask. All cell culture operations were performed in a class 2 laminar hood. Once established, cultures were visually checked

daily for media clarity. Turbidity suggested either infection or detached/dead cells. Media was replaced by fresh media every 2 days.

When cells were approximately 80-90% confluent they were re-seeded in a 1:4 ratio. Media, sterile phosphate buffered saline (PBS) and 0.25% trypsin-EDTA were warmed in a water bath to 37°C. 11ml of warm fresh media were placed in each of four T75 flasks and placed in the incubator with 5% CO_2 at 37°C. Media was then removed from the flask to be passaged and the cells washed with 8ml of warmed sterile PBS. 2ml of warmed sterile 0.25% trypsin-EDTA (GIBCO, Invitrogen, UK) was added and the flask was placed at 37°C in 5% CO_2 for 3 minutes. The flask was tapped to detach and disperse the cells and viewed under the microscope. 8ml of fresh warm media was added to the flask and the contents of the flask ie. 10ml of cell suspension was centrifuged at 1200rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 8ml fresh warm media. The four flasks with 11ml of fresh warm media were retrieved from the incubator and 2ml of cell suspension from the flask to be passaged was added to each of the four flasks. The flasks were then replaced in the incubator with 5% CO_2 at 37°C.

All experimental work was performed using ARPE-19 cells seeded onto 6 well plates (NUNC, Thermoscientific, UK). Cells were cultured in DMEM supplemented with penicillin, streptomycin and 10% foetal bovine serum until 80-90% confluent. Media was then changed to DMEM supplemented with penicillin streptomycin and 1% foetal bovine serum. Cells were maintained in

cuture with 1% foetal bovine serum for 2 weeks to allow differentiation before experiments were performed.

3.3.3 Cell storage

To ensure adequate cells were available for future use cells were frozen and stored. This was carried out when cells reached 80-90% confluence. Cells were detached with 0.25% trypsin-EDTA as decribed in section 3.3.2 and after adding media with 10% FBS, the suspension was centrifuged at 1200rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 1ml of freezing medium (10% dimethyl sulfoxide (DMSO), Fisher, UK) in FBS (GIBCO, Invitrogen, UK). 1ml samples of the suspension were then aliquoted into cryovials (NUNC, Thermo-fisher scientific, UK). The cryovials were stored at -150°C.

3.3.4 Cell thawing

When frozen cells were required for use 12ml of warmed media was added to a T75 flask and stored in the 5% CO_2 incubator at 37°C. A cryovial containing frozen cells was warmed to 37°C in a water bath until no crystals remained. The contents of the cryovial were then added to the T75 flask and placed in the 5% CO_2 incubator at 37°C. Cells were checked the next day under the microscope and media was replaced after 24 hours to ensure DMSO was completely removed from the culture media.

3.3.5 Cell counting

Performing cell counts is a recognised method for studying cell behaviour in vitro. An immediate or short term reponse, such as an alteration in membrane permeability or perturbation of a particular metabolic pathway correlates with cell proliferation or survival.

A Scepter hand held automated cell counter with 60µm disposable sensors (Merck Millipore, UK) was used to perform cell counts. The manufacture's software allowed identification of the live cell population based on particle size. Smaller particles representing dead cells and debris were distinguished and excluded from the final cell counts.

To perform cell counts, a sample of cell suspension was centrifuged at 1200rpm for 5 minutes at 4°C and the supernatant discarded. The cellular pellet was resuspended in 1ml of PBS, a 60µm sensor was attached to the Scepter cell counter and the sensor placed in the cell suspension. A reading was obtained and the live cell population identified by varying the software parameters.

3.3.6 Cell viability and apoptosis

Although the Scepter cell counter was able to broadly distinguish the live cell population from debris and dead cells, the limitations of the software did not allow identification of cells undergoing apoptosis. Hence flow cytometry was utilised with propididium iodide (PI), BD Pharmingen, UK) and FITC Annexin V (BD Pharmingen, UK). A BD Accuri™ C6 flow cytometer was used.

One part of 10X Annexin V binding buffer (BD Pharmingen, UK) containing 0.1M Hepes/NaOH (pH 7.4), 1.4 M NaCl and 25mM $CaCl_2$ was diluted with 9 parts of distilled water for a 1X working solution.

Cells were washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of 1 x 10^6 cells/ml. Then 100µl of the solution (1 X 10^5 cells) was transferred to an eppendorf tube. Following this, 5µl of FITC Annexin V and 5µl of PI was added and the cells were gently vortexed and incubated for 15 minutes at room temperature (25°C) in the dark. 400µl of 1X binding buffer was added to each tube. Analysis by flow cytometry was performed within 1 hour. Samples were analysed in triplicate. Three control groups were used for compensation: unstained cells, cells stained with FITC Annexin V only, and cells stained with PI only.

3.3.7 Glycated albumin

The effects of glycated albumin were simulated by preparing a glycated albumin solution. A 25mg vial of lyophilised glycated albumin (Sigma Aldrich, UK) was reconstituted with 2.5ml PBS to make a solution of 10mg/ml. This solution was then diluted into 2mg/ml aliquots and stored at -25°C.

For a negative control, non glycated human albumin (Sigma Aldrich, UK) was used. 50ml PBS was added to 5g of non glycated albumin to prepare a 100mg/ml solution. This solution was then diluted into 2mg/ml aliquots and stored at -25°C.

ARPE-19 cells were cultured in T75 flasks (Nunc, Thermoscientific) in DMEM supplemented with 10% FBS until 80-90% confluent. Cells were then seeded at 5 x 10^4 cells per well onto 6 well plates (Nunc, Thermoscientific) and maintained in culture until 80-90% confluent in a humidified incubator at 37°C with 5% CO_2. Media was then replaced with serum free media (SFM) for 16 hours prior to experiments.

Once defrosted, 500µg samples of diluted glycated albumin were added to each well of a six well plate. 500µg samples of diluted albumin were used as negative controls and cell counts and analysis with flow cytometry was performed after 24 hours as described in sections 3.3.5 and 3.3.6.

3.3.8 Positive controls: Interleukin 1-β and tumour necrosis factor-α

Interleukin 1-β (IL-1β, Merck Millipore, UK) and tumour necrosis factor α (TNF-α, Merk Millipore, UK) were used as positive controls to stimulate the production of MCP-1 and IL-8 in ARPE cells.

A vial containing 10µg of recombinant human interleukin-1β (Merck Millipore, UK) was reconstituted with 100µl of distilled water to yield a dilution of 100ng/µl.

This was then aliquoted into 2µl (200ng) samples and stored at -20°C until required. When required, a sample was defrosted and diluted by addition of 18µl of PBS to yield a concentration of 10ng/µl.

A vial containing 50µg of recombinant human TNF-α (TNF-α, Merk Millipore, UK) was reconstituted with 100µl of distilled water yielding a concentration of

500ng/µl. Aliquots of 4µl (2000ng) were stored at -20°C. For use, aliquots were defrosted and diluted with 396µl of PBS equivalent to a concentration of 20ng per sample.

3.3.9 Inhibitor preparations

Ly294002 (Sigma Aldrich, UK), a specific inhibitor of the phosphatidylinositol-3-kinase (P13K) pathway was used. 1mg was added to 29ml of dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK) resulting in a solution of 100µM/ml.

500µl of this solution equivalent to 50µM was used to treat ARPE-19 cells.

SB202190 (Sigma Aldrich, UK) a p38 inhibitor was prepared by adding 5mg to 50ml DMSO (Sigma Aldrich, UK) resulting in a solution of 300µM/ml.

100µl of this solution equivalent to 30µM was used to treat ARPE-19 cells

JSH-23 (Sigma Aldrich, UK) a NF-κB p65 subunit inhibitor was used.

5mg was added to 41.5ml of DMSO (Sigma Aldrich, UK) to prepare a solution of 500µM/ml

100µl of this solution equivalent to 50µM was used to treat ARPE-19 cells.

3.4 Hypoxia

For hypoxic experiments a second incubator (Sanyo, Panasonic, USA) was used. Cells were mainatained at 37°C in a humidified environment with 5% CO_2. Nitrogen was used to diplace oxygen to generate various hypoxic states.

Cells were maintained in hypoxia (2% oxygen) for 24 or 48 hours prior to experiments.

3.4.1 Experimental design

Minocycline (Sigma Aldrich, UK) was used for experimental work. 0.1g was dissolved in 5ml of PBS. 0.5ml of this solution was diluted 1:2 to prepare a 10mM concentrated solution. This was diluted 1:10 for a working solution of 100µM/ml equivalent to 1µM in 10µl.

The first experiments were conducted to determine the effects of minocycline on the viability and growth of human RPE (ARPE-19) cells in culture.

ARPE-19 cells were thawed from frozen as described in section 2.14 and maintained in T75 flasks with DMEM supplemented with 10% FBS and penicillin streptomycin as described in section 2.12. When cells reached 80-90% confluence they were detached with trypsinisation as described in section 3.3.2

Cells were reseeded at 5 x 10^4 per well in 6 well plates and the next day media was replaced with serum free media for 16 hours prior to addition of varying concentrations of minocycline. Cells were exposed to minocycline for 24 hours, then the media was discarded and the cells were detached with trypsin and resuspended in 1 ml of PBS for cell counts measured with the Scepter handheld automated cell counter.

For construction of cell growth curves, eight 6 well plates were used. In each of the eight plates 3 wells were seeded with 5 x 10^4 cells per well. Cell counts

were performed in triplicate on eight successive days using the Scepter cell counter.

To investigate the effects of hypoxia, IL-1β, TNF-α and minocycline on ARPE-19 cell apoptosis rates, cells were analysed with annexin V and propidium iodide staining and flow cytometry.

The effects of varying concentrations of minocycline on ARPE-19 cell viability were investigated. A concentration of minocycline that did not affect cell viability was identified.

To investigate the effects of minocycline on MCP-1 and IL-8 production from ARPE-19 cells human glycated albumin (Sigma Aldrich, UK) 500µg was added to serum free culture medium for 16 hours to induce production of the cytokines.

TNF-α (Millipore, UK) 20ng and IL-1β 20 ng (Millipore, UK) were also used to induce cytokine release from ARPE-19 cells as positive controls. TNF-α and IL-1β are well established inflammatory mediators. Human albumin (non glycated) (Sigma Aldrich, UK) was used as a negative control when added to 6 well plates in the experiments. Experiments were performed in normoxic conditions where cells were placed in a 5% CO_2 incubator at 37°C and also in hypoxic conditions where cells were placed in a hypoxic incubator with 2% O_2, 5% CO_2. Experiments with minocycline added as pretreatment to serum free media prior to stimulants was performed. Minocycline was also added to serum free media after 16 hours of exposure to stimulants to investigate treatment effect.

The supernatant was then collected and stored at -80°C. The cells were lysed with a cell lysis buffer and also stored at -80°C.

Samples were then defrosted and analysed by enzyme linked immunosorbent assay. A brief description of the technique is described below, a detailed protocol is provided in Appendix A.

For IL-8, a solid phase sandwich enzyme linked immunosorbent assay (ELISA) was used (Invitrogen, UK). A monoclonal antibody specific for human IL-8 had been coated onto the wells of a microtiter strip provided. Samples, including standards of known human IL-8 content, control specimens, and unknowns, were pipetted into these wells followed by the addition of a second biotinylated monoclonal antibody.

During the primary incubation, the human IL-8 antigen binds to the immobilized (capture) antibody on one site and to the solution phase biotinylated antibody on a second site.

After removal of excess second antibody, streptavidin- peroxidase (enzyme) was added. This bound to the biotinylated antibody to complete the four member sandwich. After a second incubation and washing to remove the unbound enzyme, a substrate solution was added, which was acted upon by the bound enzyme to produce colour. The intensity of the coloured product is directly proportional to the concentration of human IL-8 present in the original specimen. The intensity of colour was measured at 450nm utilising a Varoskan Flash platereader (Thermoscientific, UK).

A similar process was used for MCP-1 ELISA (Abcam, UK). During this assay an antibody specific for human MCP-1 coated on a 96 well plate was used. Standards and samples were pipetted into the wells and MCP-1 present in a sample was bound to the wells by the immobilised antibody. The wells were washed and biotinylated antihuman MCP-1 antibody added. After washing away unbound biotinylated antibody, HRP –conjugated streptavidin was pipetted into the wells. The wells were again washed, a 3, 3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to the wells and colour developed in proportion to the amount of human MCP-1 present in the original specimen. Stop solution was then added to the wells and the colour changes from blue to yellow. The intensity of the colour was measured at 450nm with a Varoskan Falsh platereader (Thermoscientific, UK)

3.4.2 Statistical analysis

Data were analysed with graphpad prism, version 6.2 (Graphpad, CA, USA).

Data for cell viability and apoptosis were analysed utilising a non-parametric approach using a Kruskal-Wallis test. This approach was used as it was assumed that the results would be skewed and not normally distributed. It was assumed that the results in each experimental group would have the same distribution which is a limitation of the test. Kruskal Wallis [259] tests whether the probability that a random observation from each group is equally likely to be above or below a random observation from another group.The data quantity used in the comparison is the median of all pairwise differences.

Results from IL-8 and MCP-1 ELISA were analysed with a one way ANOVA as a comparison between more than two experimental groups was utilised. The model assumes data is normally distributed, and the one way ANOVA is considered a robust test against the normality assumption. Tukey's test[260] was used in conjunction with an ANOVA to compare the mean of every experimental treatment to the mean of every other treatment. It was therefore applied simulataneously to the set of all pairwise comparisons. The assumptions of the Tukey[260] test are that the groups with each mean in the test are normally distributed, there is equal within group variance across the groups associated with each mean in the test, and that the observations being tested are independent within and among the groups.

3.5 Results

3.5.1 Effects of glycated albumin and hypoxia on ARPE-19 cell growth

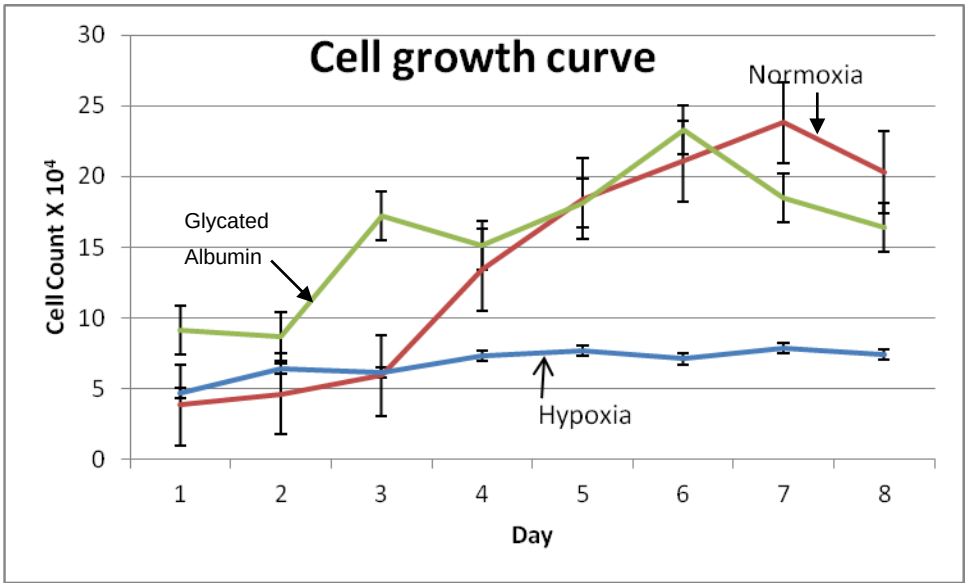

Figure 10: ARPE-19 cell growth curves in normoxia, normoxia with glycated albumin and hypoxia (2% oxygen). Each growth curve represents the results of three independent experiments performed in triplicate. Each data point represents the mean ± standard deviation.

The first series of experiments were conducted to establish a therapeutic range for minocycline on ARPE-19 cells in culture. Minocycline concentrations above 5µM adversely affected cell viability (Figure 11).

3.5.2 Concentration dependent effects of Minocycline on ARPE-19 cell viability

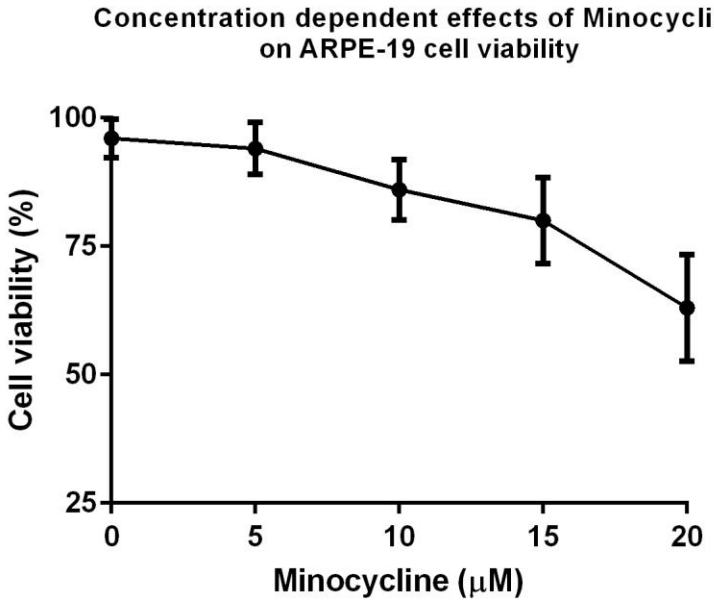

Figure 11: Concentration dependent effects of minocycline on ARPE-19 cell viability. The graph shows the percentage of non viable cells identified with propidium iodide staining utilising flow cytometry. Data shown in Appendix C, table 33, page 216

3.5.3 Effects of minocycline and hypoxia on ARPE-19 cell viability

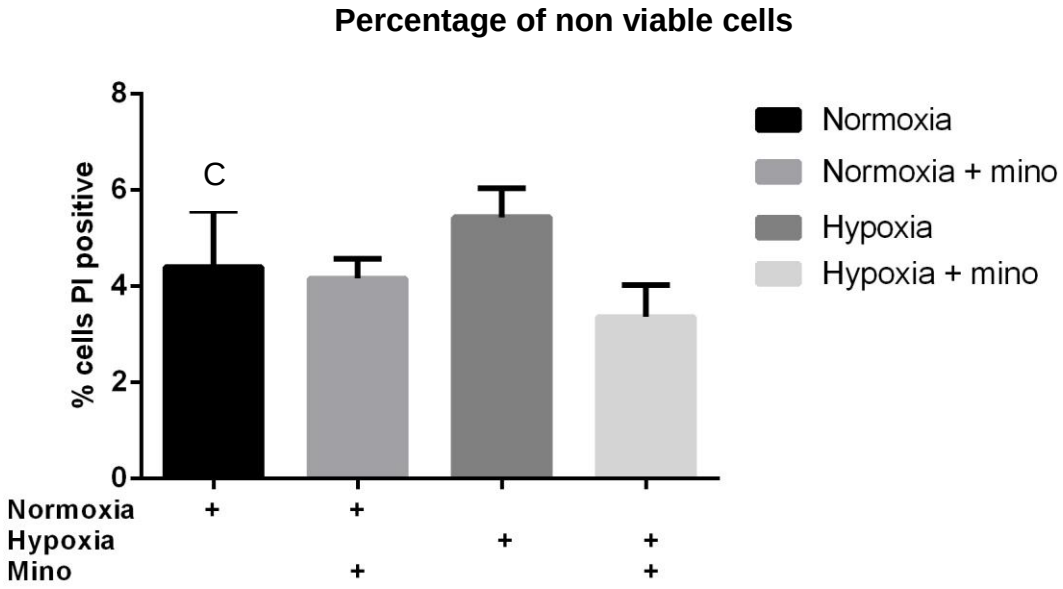

Figure 12: Proportion of cells that are non viable and prodium iodide (PI) positive after exposure to hypoxia and minocycline 5µm. Cells were exposed to each experimental condition for 24 hours. Three independent experiments were performed in triplicate for each condition investigated. Results show the mean±SD. A Krushkal wallis test did not demonstrate a difference between the four experimental conditions. C= Control
Data shown in Appendix C (page 199)

Figure 12 shows that hypoxia (2% oxygen) for 24 hours and minocycline 5µm did not adversely effect cell viability.

The next set of experiments were conducted to determine the proportion of ARPE-19 cells undergoing apoptosis under different experimental conditions. The results are shown in figure 13 below.

3.5.4 Effects of minocycline and hypoxia on apoptosis

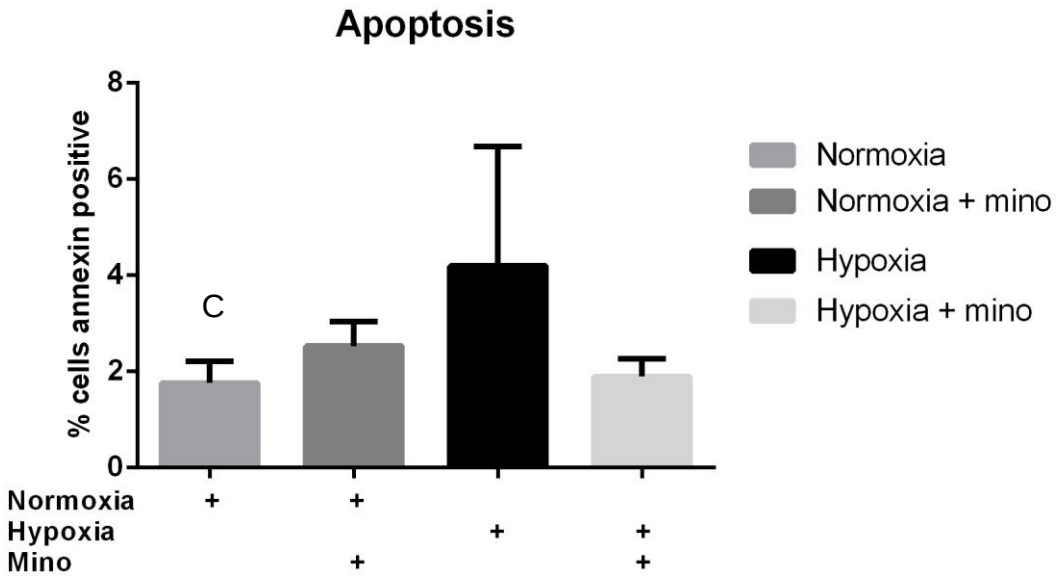

Figure 13: Proportion of cells undergoing apoptosis and annexin V positive after exposure to hypoxia and minocycline 5µm. Cells were exposed to each experimental condition for 24 hours. Three independent experiments were performed in triplicate for each condition investigated. Results show the mean±SD. A Krushkal wallis test did not demonstrate a difference between the four experimental conditions. C = Control
Data shown in Appendix C (page 200)

Figure 13 shows that hypoxia (2% oxygen) for 24 hours and minocycline 5µm did not increase ARPE-19 apoptosis rates.

3.5.5 Effects of glycated albumin on ARPE-19 cell viability after exposure to minocycline and hypoxia.

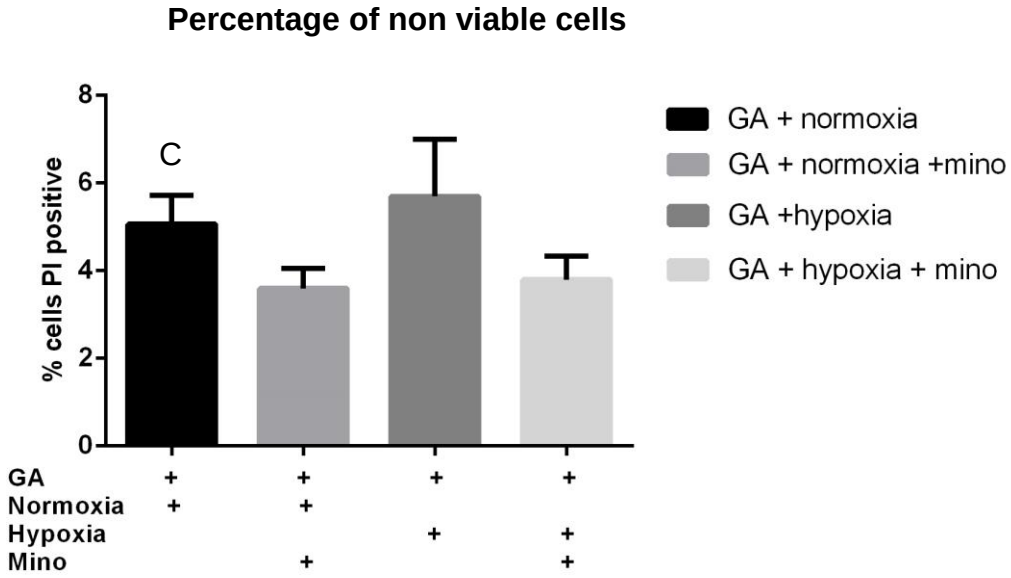

Figure 14: Proportion of cells that are non viable and prodium iodide (PI) positive after exposure to glycated albumin, hypoxia and minocycline 5µm. Cells were exposed to each experimental condition for 24 hours. Three independent experiments were performed in triplicate for each condition investigated. Results show the mean±SD. A Krushkal wallis test did not demonstrate a difference between the four experimental conditions.
C= Control. Data shown in Appendix C (page 201)

Figure 14 shows cell viability did not differ when ARPE-19 cells were exposed to different conditions of glycated albumin, in combination with minocycline 5µm or hypoxia (2% oxygen).

To investigate the effects of glycated albumin on apoptosis after exposure to minocycline and hypoxic conditions further experiments were conducted, the results of which are shown in figure 15.

3.5.6 Effects of glycated albumin on apoptosis after exposure to minocycline and hypoxia.

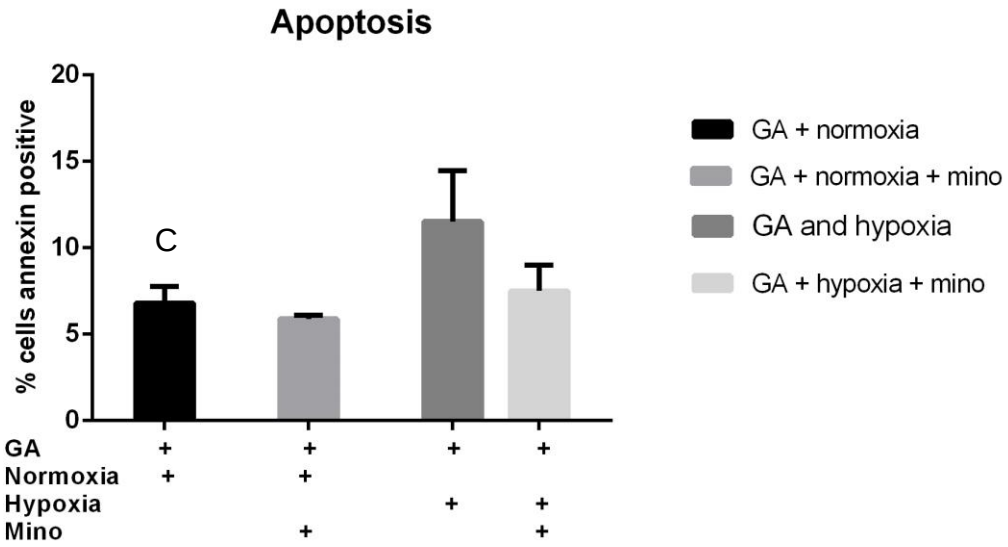

Figure 15: Proportion of cells undergoing apoptosis and annexin V positive after exposure to glycated albumin, hypoxia and minocycline 5µm. Cells were exposed to each experimental condition for 24 hours. Three independent experiments were performed in triplicate for each condition investigated. Results show the mean±SD. A Krushkal wallis test did not demonstrate a difference between the four experimental conditions. C=Control. Data shown in Appendix C (page 202)

Figure 15 shows that apoptosis rates did not differ when ARPE-19 cells were exposed to experimental conditions of glycated albumin, combined with hypoxia (2% oxygen) for 24 hours and minocycline 5µm.

Increased doses of minocycline above 5µM and increasing the length of exposure to hypoxia (2% O_2) to 48 hours resulted in the majority of cells becoming non viable. Therefore, further hypoxic experiments were performed with 24 hours exposure to 2% oxygen and minocycline concentrations of 5µM

were used. These conditions were used to investigate IL-8 and MCP-1 cytokine production from ARPE-19 cells and the effects of minocycline.

3.6 IL-8 ELISA

A standard curve was constructed from the results of duplicate standard dilutions as per instructions provided by the manufactor (Invitrogen, UK). The absorbance of the standards against the standard concentrations was plotted on a logarithmic scale.

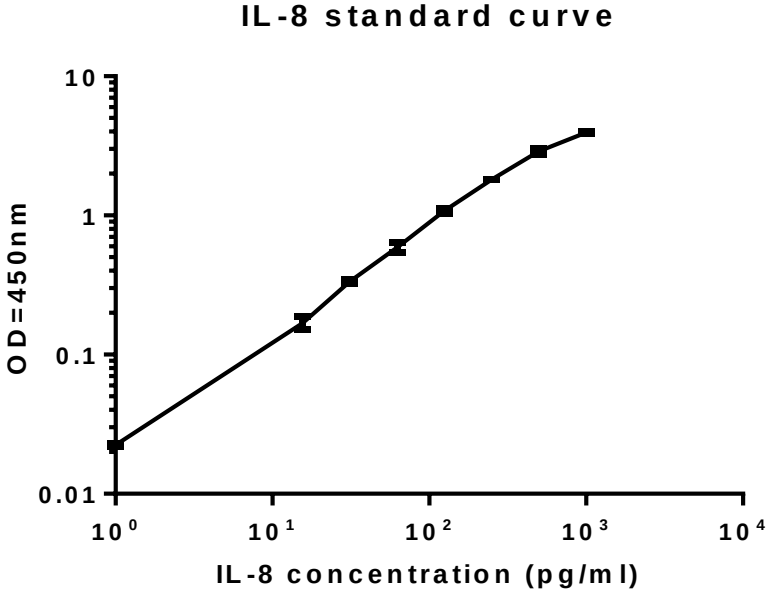

Figure 16: IL-8 standard curve Results show Optical densities (OD) measured at 450nm plotted against IL-8 concentration in picogrammes per millilitre (pg/ml). Results are plotted using a logarithmic scale. Each point represents the mean±standard deviation of standard dilutions of IL-8 measured in duplicate.

3.6.1 Reproducibility of IL-8 ELISA

Intraplate and interplate reproducibility tests were performed. A pair of positive and negative controls were tested 30 times in the same plate (intraplate) and

this was then repeated in different plates (interplate) on different dates. Mean, standard deviation and coefficient of variation were calculated from both interplate and intraplate optical densities (ODs). Results are shown in table 2 below.

Table 2: Reproducibility of IL-8 ELISA

		Intraplate precision			**Interplate precision**	
IL-8	**n**	**OD(Mean±SD)**	**CV(%)**	**N**	**OD(Mean±SD)**	**CV(%)**
0 pg/mL	30	1.176±0.088	7.4	30	1.301±0.391	30.1
1000pg/mL	30	4.74±0.691	14.6	30	4.934±0.369	7.5

The IL-8 concentrations from unknown samples were calculated using graphpad prism version 6.2 analysis software (Graphpad software Inc. San Diego California, USA).

The results of flow cytometry presented in figures 12 to 15 earlier in this chapter did not demonstrate a statistical difference in numbers of viabile cells or apoptosis rates when ARPE-19 cells were exposed to various combinations of hypoxia (2% oxygen) for 24 hours, glycated albumin 500µg/ml or minocycline 5µM. Therefore, these conditions were selected to investigate the effects of hypoxia, glycated albumin and minocycline on IL-8 and MCP-1 production utilising enzyme linked immunosorbant asssy (ELISA).

3.6.2 Effects of hypoxia and minocycline on glycated albumin induced IL-8 production from ARPE-19 cells

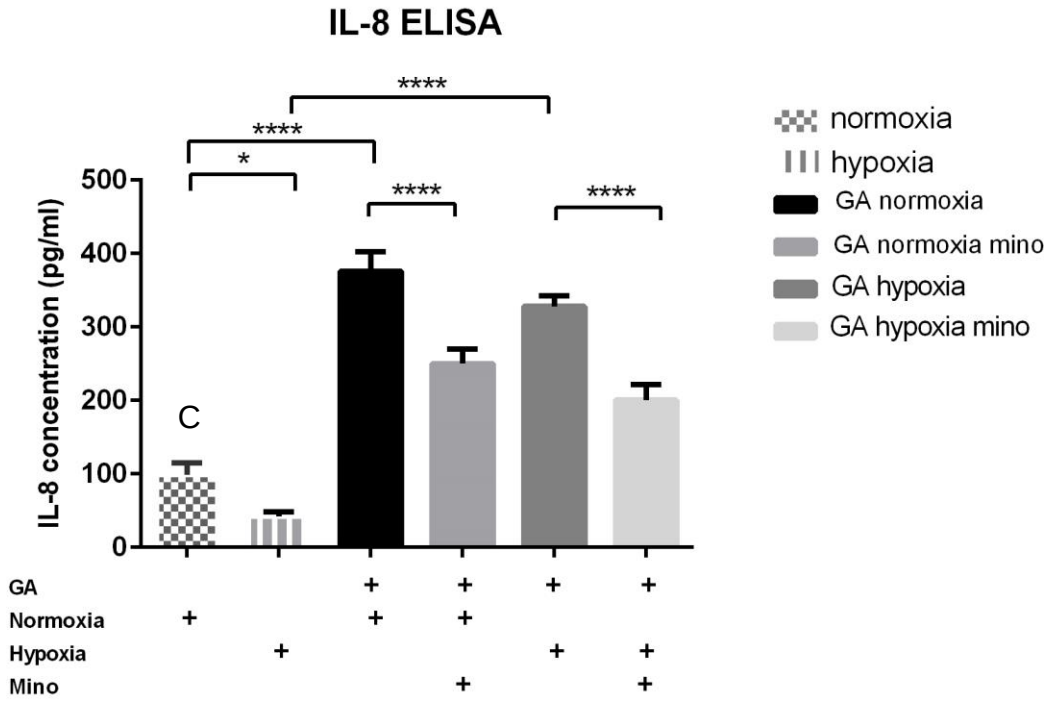

Figure 17: The figure above shows the results of exposure of ARPE-19 cells to four experimental conditions, glycated albumin (GA) and normoxia; GA, normoxia and minocycline 5μM; GA and hypoxia; and GA hypoxia and minocycline 5μM. The results shown are the mean ± standard deviation of each experimental conditions repeated in duplicate. One way ANOVA and Tukey's multiple comparions test was used to assess differences between experimental groups. Statistical significance is indicated in the results shown as ** (p < 0.0001), *** (p = 0.0001 to 0.001) and ** (p = 0.001 to 0.01)**
C= Control. Data shown in Appendix C (page 203)

The results from Figure 17 show that there was a statistically significant decrease in glycated albumin stimulated IL-8 production from ARPE-19 cells associated with minocycline. This effect occurred in both normoxic and hypoxic experimental conditions.

3.6.3 Effects of minocycline on IL-1β stimulated IL-8 production in normoxia and hypoxia

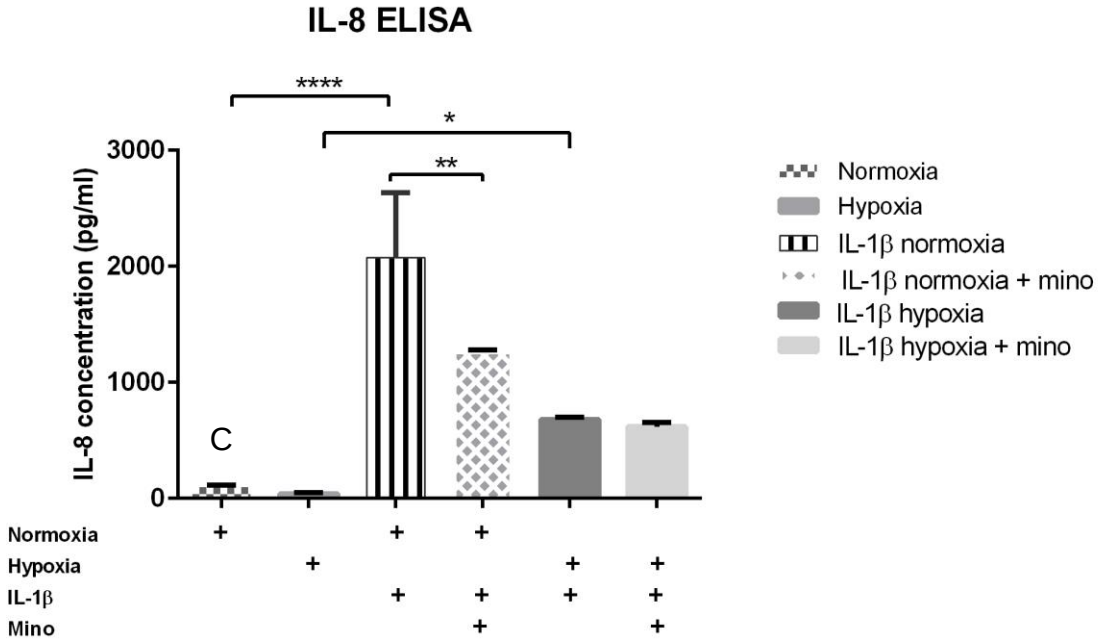

Figure 18: The figure above shows the results of exposure of ARPE-19 cells to four experimental conditions, IL-1β and normoxia; IL-1β, normoxia and minocycline 5μM; IL-1β and hypoxia; and IL-1β, hypoxia and minocycline 5μM. The results shown are the mean±standard deviation of each experimental conditions repeated in duplicate. One way ANOVA and Tukey's multiple comparisons test was used to assess differences between experimental groups. Statistical significance is indicated in the results shown as * (p = 0.0001 to 0.001), ** (p = 0.001 to 0.01) and * (p = 0.01 to 0.05) C=Control**
Data shown in Appendix C (page 205)

The results in Figure 18 show that minocycline was associated with a statistically significant decrease in IL-1β induced IL-8 production from ARPE-19 cells in normoxic conditions but not in hypoxia.

3.6.4 Effects of individual biochemical inhibitors on glycated albumin stimulated IL-8 production from ARPE-19 cells in normoxia and hypoxia

The exact mechanisms by which minocycline may produce abrogation of IL-8 production from glycated albumin stimulated ARPE-19 cells remains unclear. Biochemical pathway inhibitors, Ly294002, SB202190 and JSH-23 were therefore studied individually and in combination and the results are shown in figures 19 and 20.

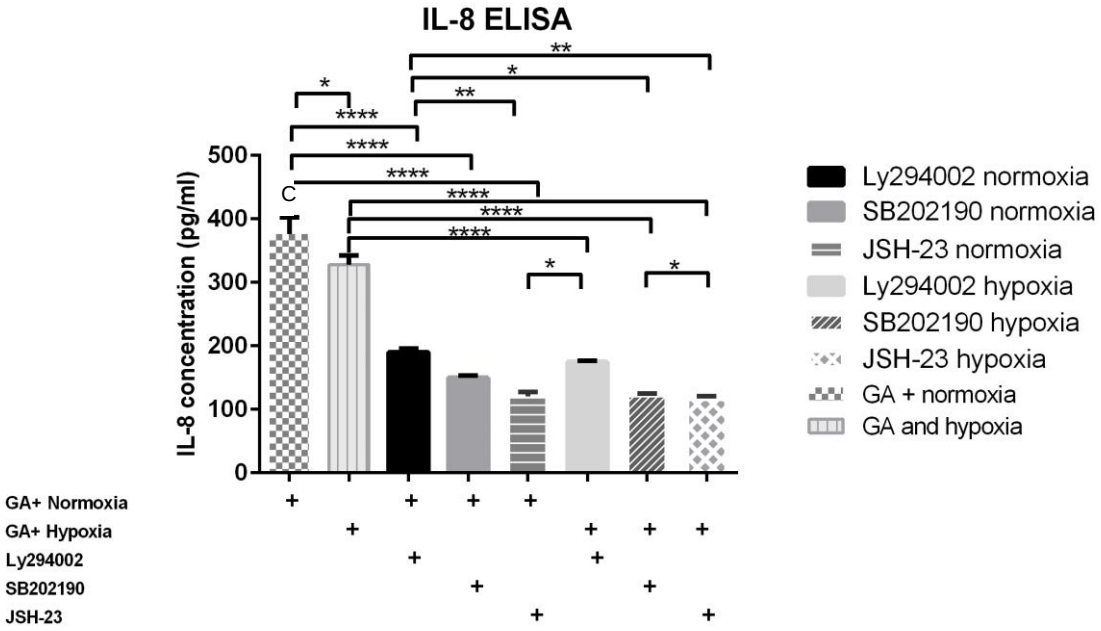

Figure 19: The figure above shows the results of exposure of ARPE-19 cells to six experimental conditions: (1) glycated albumin (GA), Ly294002 and normoxia; (2) GA, SB202190 and normoxia; (3) GA, JSH-23 and normoxia; (4) GA, hypoxia and Ly294002; (5) GA, hypoxia and SB202190 and (6) GA, hypoxia and JSH-23. The results shown are the mean ± standard deviation of each experimental condition repeated in duplicate. One way ANOVA and Tukey's multiple comparisons test was used to assess differences between experimental groups. Statistical significance is indicated in the results shown as ⁎⁎⁎⁎ (p = < 0.0001), ⁎⁎⁎ (p = 0.0001 to 0.001), ⁎⁎ (p = 0.001 to 0.01) and ⁎ (p = 0.01 to 0.05)
C = Control. Data shown in Appendix C (page 206)

The results in Figure 19 show individual biochemical inhibitors all suppressed glycated albumin induced IL-8 production both in normoxic and hypoxic conditions.

3.6.5 Effects of combination of biochemical inhibitors on glycated albumin induced IL-8 production

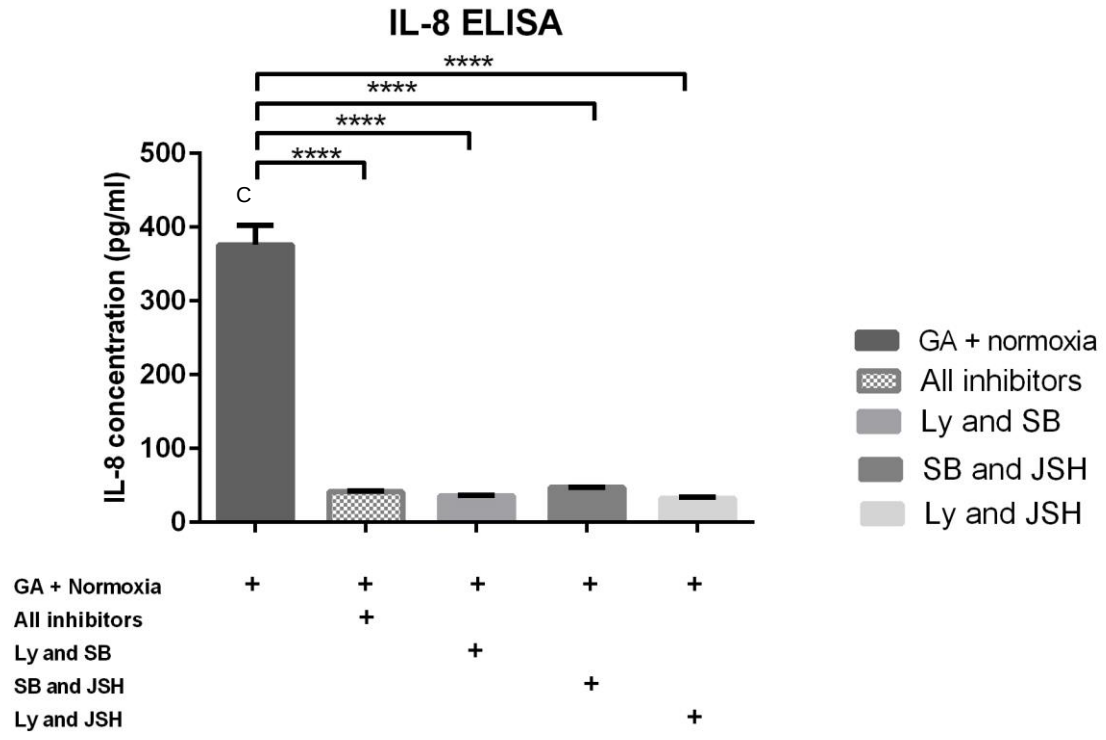

Figure 20: The figure above shows the results of exposure of ARPE-19 cells to four experimental conditions: (1) glycated albumin (GA), Ly294002, SB202190 and JSH-23; (2) GA, Ly294002 and SB202190; (3) GA, SB202190 and JSH-23; (4) GA, Ly294002 and JSH-23. The results shown are the mean ± standard deviation of each experimental condition repeated in duplicate. One way ANOVA and Tukey's multiple comparisons test was used to assess differences between experimental groups. Statistical significance is indicated in the results shown as * (p = 0.0001 to 0.001), ** (p = 0.001 to 0.01) and * (p = 0.01 to 0.05)**
C= Control. Data shown in Appendix C (page 208)

The results shown in Figure 20 show that biochemical inhibitors in combination all demonstrate a statistically significant decrease in glycated albumin induced IL-8 production from ARPE-19 cells.

3.7 MCP-1 ELISA

A standard curve was constructed from the results of duplicate standard dilutions as per instructions provided by the manufactor (Invitrogen, UK). The absorbance of the standards against the standard concentrations was plotted on a logarithmic scale.

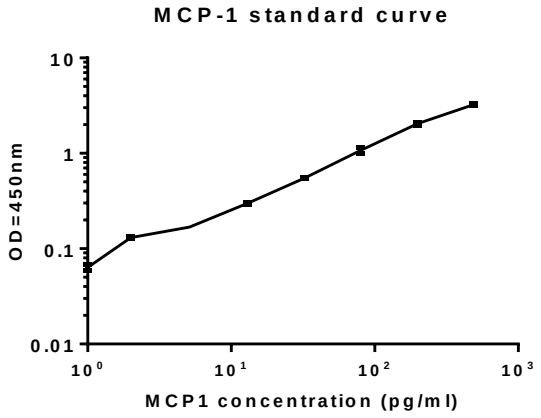

Figure 21: MCP-1 standard curve. Results show Optical densities (OD) measured at 450nm plotted against MCP-1 concentration in picogrammes per millilitre (pg/ml). Results are plotted using a logarithmic scale. Each point represents the mean±standard deviation of standard dilutions of MCP-1 measured in duplicate.

3.7.1 Reproducibility of MCP-1 ELISA

Intraplate and interplate reproducibility tests were performed. A pair of positive and negative controls were tested 30 times in the same plate (intraplate) and

this was then repeated in different plates (interplate) on different dates. Mean, standard deviation and coefficient of variation were calculated from both interplate and intraplate optical densities (ODs). Results are shown in table 3 below.

Table 3: Reproducibility of MCP-1 ELISA

		Intraplate precision			Interplate precision	
MCP-1	**n**	**OD(Mean±SD)**	**CV(%)**	**n**	**OD(Mean±SD)**	**CV(%)**
0 pg/mL	30	1.593±0.148	9.3	30	1.188±0.223	18.8
1000pg/mL	30	4.098±0.271	6.61	30	4.267±0.314	7.4

3.7.2 Effects of hypoxia and minocycline on glycated albumin induced MCP-1 production

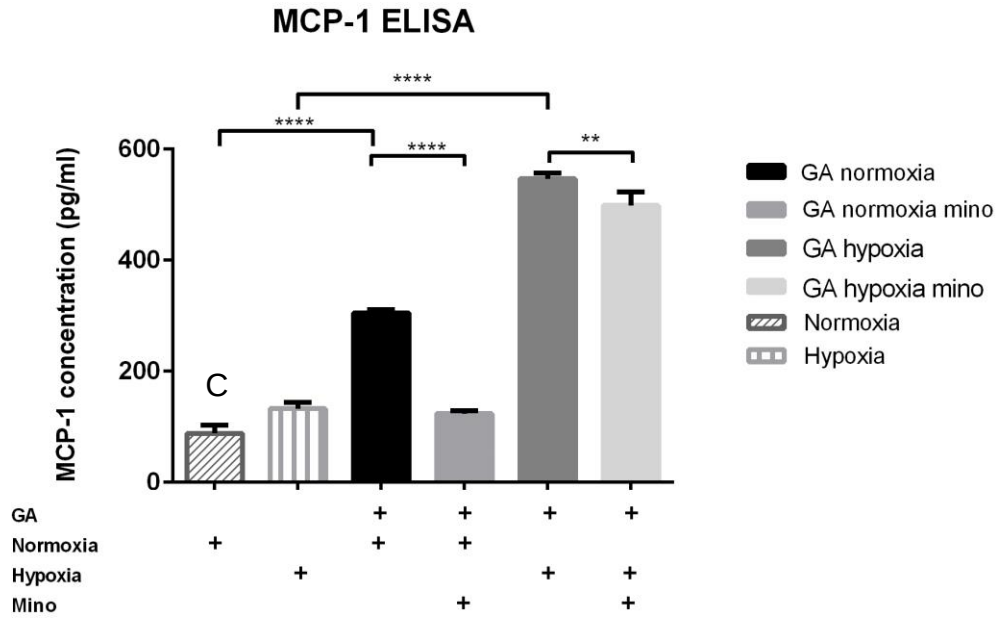

Figure 22: The figure above shows the results of exposure of ARPE-19 cells to four experimental conditions, (1) glycated albumin (GA) and normoxia; (2) GA, normoxia and minocycline 5μM; (3) GA and hypoxia; and (4) GA, hypoxia and minocycline 5μM. The results shown are the mean ± standard deviation of each experimental conditions repeated in duplicate. One way ANOVA and Tukey's multiple comparions test was used to assess differences between experimental groups. Statistical significance is indicated in the results shown as ** (p < 0.0001), and ** (p = 0.001 to 0.01) C= Control. Data shown in Appendix C (page 210)**

The results in Figure 22 show that minocycline statistically significantly decreased glycated albumin induced MCP-1 production. The effects were greater in normoxic conditions compared to hypoxia. Hypoxia increased glycated albumin induced MCP-1 production.

3.7.3 Effects of minocycline on IL-1β induced MCP-1 production in normoxia and hypoxia

One way ANOVA and Tukey's multiple comparisons test was used to assess the differences between experimental groups. No meaningful statistical differences were demonstrated. The data and related statistical analysis is in Appendix C (tables 31 and 32, page 215).

3.7.4 Effects of individual biochemical inhibitors on glycated albumin stimulated MCP-1 production from ARPE-19 cells in normoxia and hypoxia

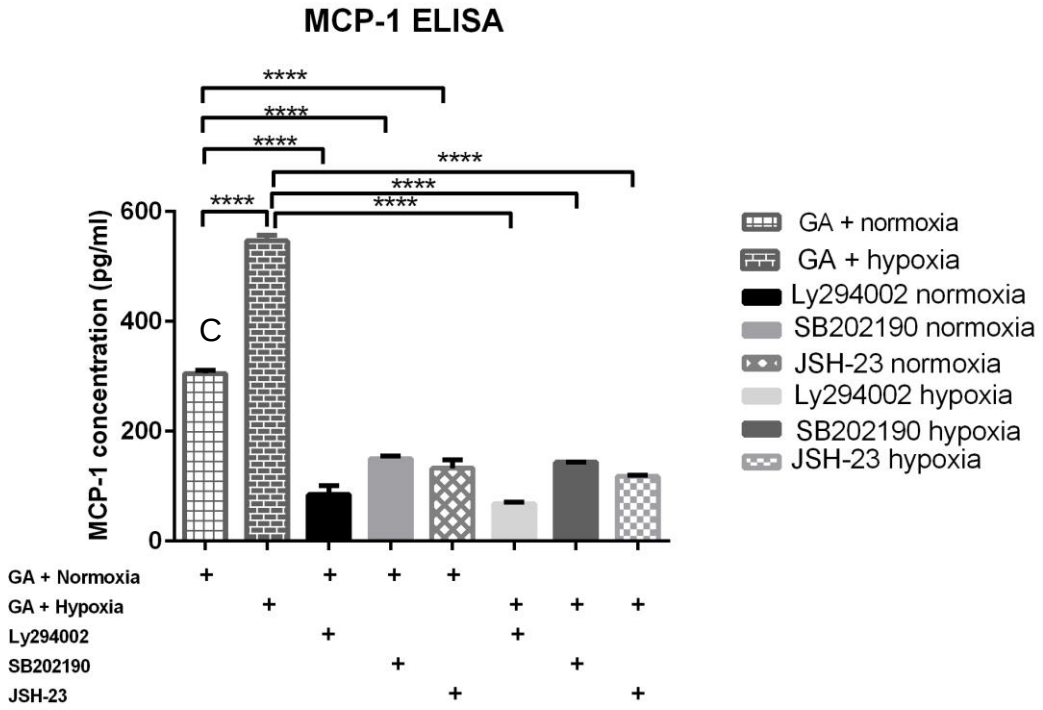

Figure 23: The figure above shows the results of exposure of ARPE-19 cells to six experimental conditions: (1) glycated albumin (GA), Ly294002 and normoxia; (2) GA, SB202190 and normoxia; (3) GA, JSH-23 and normoxia; (4) GA, hypoxia and Ly294002; (5) GA, hypoxia and SB202190 and (6) GA, hypoxia and JSH-23. The results shown are the mean ± standard deviation of each experimental condition repeated in duplicate. One way ANOVA and Tukey's multiple comparisons test was used to assess differences between experimental groups. Statistical significance is indicated in the results shown as ** ($p < 0.0001$) C=Control. Data shown in Appendix C (page 212)**

Individual biochemical inhibitors all statistically significantly decreased glycated albumin induced MCP-1 production from ARPE-19 cells.

3.7.5 Effects of combination of biochemical inhibitors on glycated albumin induced MCP-1 production

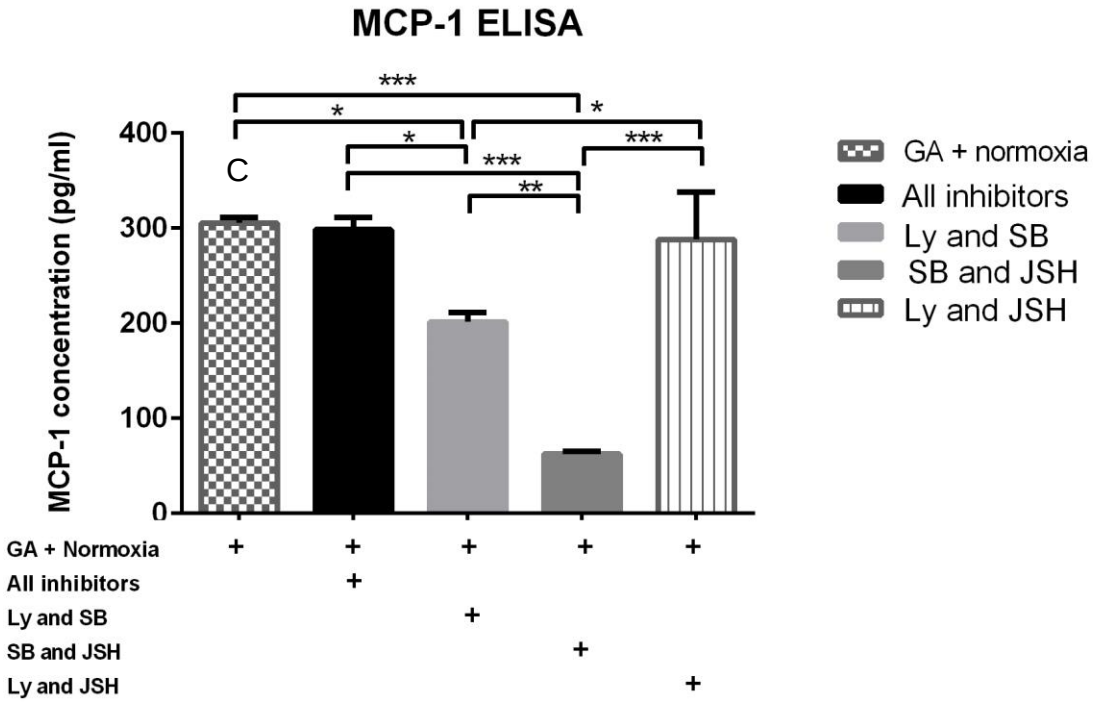

Figure 24: The figure above shows the results of exposure of ARPE-19 cells to four experimental conditions: (1) glycated albumin (GA), Ly294002, SB202190 and JSH-23; (2) GA, Ly294002 and SB202190; (3) GA, SB202190 and JSH-23; (4) GA, Ly294002 and JSH-23. The results shown are the mean ± standard deviation of each experimental condition repeated in duplicate. One way ANOVA and Tukey's multiple comparisons test was used to assess differences between experimental groups. Statistical significance is indicated in the results shown as * (p = 0.0001 to 0.001), ** (p = 0.001 to 0.01) and * (p = 0.01 to 0.05)**
C= Control. Data shown in Appendix C (page 214)

The results in Figure 24 show that a combination of biochemical inhibitors varied in the response to suppression of MCP-1 production from ARPE-19 cells. SB202190 in combination with JSH-23 was most effective at suppressing glycated albumin induced MCP-1 production.

3.8 Discussion

3.8.1 Effects of glycated albumin and hypoxia on ARPE-19 cell growth

The results of the growth curves in figure 10 show that glycated albumin (500µg/ml) did not impair cell growth. Hypoxia caused a flattening of the growth curve. This suggests that hypoxia acts by inhibition of cell division, or a combination of inhibited cell division and promotion of cell loss by inducing apoptotis or necrosis.

The cell growth curves were constructed after cell counts were obtained after culturing ARPE-19 cells in media containing 10% foetal bovine serum. In physiological conditions, RPE cells are non dividing cells that form the external blood retinal barrier between the neural retina and the choroidal circulation. In vivo, RPE cells are not directly exposed to serum as Bruch's membrane forms a barrier between the RPE cell layer and the blood within the choroidal vasculature. As such, measurement of cell growth parameters may be considered not directly applicable to conditions in vivo. However, in pathophysiological inflammatory conditions, such as autoimmune uveitis, RPE cells are activated and participate in recruitment and stimulation of inflammatory cells into the eye. [261] In neovascular AMD, a low grade inflammatory response is suggested to be the start of the pathophysiological process. This may progresses through recruitment of inflammatory cells and release of cytokines, the pathological endpoint being choroidal neovascularisation extending into the subretinal space and retina.

3.8.2 Effects of minocycline and hypoxia on cell viability

Figure 12 showed that flow cytometry did not demonstrate statistical differences in cells staining propidium iodide positive when exposed to hypoxia (2% oxygen) for 24 hours or minocycline 5µM. Udono et al [262] reported that hypoxia decreased ARPE-19 cell viability by approximately 20% compared to normoxic conditions. However, the hypoxic conditions used in their study were 1% oxygen compared to 2% oxygen used in this study. Also, their method[262] of cell counting used a modified 3-(4,5-dimetylthiazone-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay measures the ability of cells to reduce the tetrazolium dye, MTT to its insoluble formazan, resulting in a purple colour. As this effectively requires functioning mitochondria, the MTT effectively measures the metabolic activity of live cells. This does not necessarily correlate with cell viability. Some cells may be viable but not necessarily very metabolically active. The metabolic status of cells cultured in hypoxia is likely to be different to normoxic conditions therefore rendering the MTT assay as a measure of cell viability unreliable. In this study, it was therefore decided to use a technique that directly measured cell necrosis and apoptosis. Udono et al[260] used the modified TdT-mediated dUTP nick end labelling (TUNEL) method. This detects DNA fragmentation representing late stage apoptosis. It is unclear from their study [262] how the methodology used to detect cells undergoing apoptosis differentiated necrotic cells. Therefore, in this study, annexin V and propidium iodide staining were used to distinguish apoptotic and necrotic cells.

3.8.3 Assessment of cell viability

The method used to determine cell viability and so the definition of viability is often related to the phenomenon studied. Cell viability may be judged by morphological changes or by changes in membrane permeability and/or physiological state inferred from the exclusion of certain dyes or the uptake and retention of others. [263]

Live cells with intact membranes are distinguished by their ability to exclude dyes that easily penetrate dead or damaged cells. Staining of nonviable cells with propidium iodide (PI) has been performed on most cell types. The stained cells are bright red and easy to identify. [263]

The basal level of apoptosis and necrosis varies considerably within a population. Thus, even in the absence of induced apoptosis, most cell populations contain a minor percentage of cells that are positive for apoptosis (FITC Annexin V positive, PI negative or FITC Annexin V positive, PI positive).[263]

The untreated population is used to define the basal level of apoptotic and dead cells. Since cell death is the eventual outcome of cells undergoing apoptosis, cells in the late stages of apoptosis will have a damaged cell membrane and stain positive for PI as well as for FITC Annexin V. Thus, the assay does not distinguish between cells that have already undergone an apoptotic cell death and those that have died as a result of necrotic pathway, because in either case

the dead cells will stain with both FITC Annexin V and PI. [263] This represents one on the limitations of the methodology used in this study.

3.8.4 Differences in vitro and in vivo

Many of the differences in cell behaviour between cultured cells and their counterparts in vivo stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost, and as the cells spread out, become mobile, and start to proliferate, so the growth fraction of the cell population increases. [263]

The culture environment also lacks the several systemic components involved in homeostatic regulation in vivo, prinicipally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant in vitro than in vivo, but may not be truly representative of the tissue from which the cells were derived. Energy metabolism in vitro occurs largely by glycolysis, and although the citric acid cycle is still functional, it plays a lesser role. [263]

3.8.5 Pharmacokinetics

The measurement of toxicity in vitro is generally a cellular event. It is very difficult to recreate the complex pharmacokinetics of drug exposure in vitro, and between in vitro and in vivo experiments there usually are significant differences in exposure time to and concentration of the drug, rate of change of the concentration, metabolism, tissue penetration, clearance, and excretion.[264] It may be possible to simulate these parameters, for example using multicellular

tumour spheroids for drug penetration or timed perfusion to simulate concentration and time (C x T) effects- most studies concentrate on a direct cellular response, therby gaining simplicity and reproducibility.[262] This approach was utilised in this study.

A toxic response in vitro may be measured by changes in cell survival or metabolism. [264] This may not accurately reflect the situation in vivo which may be a tissue response (eg. inflammatory reaction, fibrosis, kidney failure) or a systemic response (eg. pyrexia, vascular dilatation). This is a limitation of the work presented in this thesis.

3.8.6 Conclusions of cell viability and apoptosis experiments

ARPE-19 cells were exposed to varying concentrations of minocycline ranging from 1µM to 20µM as previous studies [265] have indicated a potential narrow therapeutic window for minocyline. Doses higher than 5µM affected ARPE-19 cell viability. Hollborn et al [264] investigated the effects of minocycline on the viability and physiological properties of human ARPRE cells. Cell viability was determined by trypan blue assay. Their results [265] showed that minocycline at high concentrations (above 5µM) decreased the viability of human RPE cells through induction of cell necrosis. Assessment of cell viability under the microscope utilising trypan blue and cell counting with haemocytometers is a widely used technique. Viable cells have intact membranes and exclude the dye. Limitations of this method are that the technique of cell counting is time consuming, and hence prone to errors with impact on the accuracy of the results. After staining with trypan blue cell counts are usually performed within 5

minutes. This is because on standing, some viable cells may become permeable and take up the dye. This may result in some cells being counted as falsely non viable.

In this study, because of these limitations, trypan blue staining was not utilised. Whilst there are limitations of an vitro approach, the results of this study demonstrate that concentrations of minocycline below 5µm do not have an adverse effect on ARPE-19 cell viability and apoptosis. The results of this study utilising 2% oxygen for 24 hours to simulate hypoxic effects. The effects of hypoxia in this study did not demonstrate a significant decrease in ARPE-19 cell viability or increase in apoptosis suggesting that this cell line is relatively resistant to hypoxia.

3.8.7 Effects of hypoxia, minocycline and biochemical inhibitors on GA induced IL-8 production from ARPE-19 cells

The results showed that minocycline abrogated glycated albumin induced IL-8 production from ARPE-19 cells under both normoxic and hypoxic conditions. Minocycline decreased IL-1β stimulated IL-8 production in normoxia but not in hypoxic conditions. With regard to biochemical inhibitors, the effects of Ly294002, SB202190 and JSH-23 individually were no different in suppressing IL-8 production in normoxia compared to hypoxia. JSH-23 supressed IL-8 production more than SB202190 and JSH-23 in both normoxic and hypoxic conditions. Ly294002 did not suppress glycated albumin induced IL-8 production to the same extent as SB202190 and JSH-23.

In terms of various combinations of biochemical inhibitors, these experiments were all conducted in normoxia as hypoxic conditions did not show significant differences between individual inhibitors in suppressing IL-8 production. Ly294002 in combination with JSH-23 inhibited glycated albumin induced IL-8 production more than a combination of all three inhibitors.

Biochemical inhibitors, whether used individually or in combination suppressed glycated albumin induced IL-8 production more than minocycline.

3.9.7 Effects of hypoxia, minocycline and biochemical inhibitors on GA induced MCP-1 production from ARPE-19 cells

Hypoxia increased glycated albumin induced MCP-1 production. Minocycline suppressed this response, however, minocycline's effect on reducing induced MCP-1 production was greater in normoxic conditions.

Ly294002 inhibited glycated albumin induced MCP-1 production more than SB202190 and JSH-23 in normoxia and hypoxia. When biochemical inhibitors were combined JSH-23 and SB202190 had the most effect on reducing MCP-1 levels.

Use of the biochemical inhibitor Ly294002 inhibited glycated albumin induced MCP-1 production more than minocycline, however, the other biochemical inhibitors when used individually in normoxia or hypoxia elicited a similar effect to minocycline. In combination, only SB202190 and JSH-23 together had a greater effect than minocycline suppressing glycated albumin induced MCP-1 production.

3.9.8 Biochemical pathways for IL-8 and MCP- 1 production

When exposed to GHSA, hRPE cells actively secrete IL-8 and MCP-1.[266] Bian et al[266] found that AGE does not stimulate hRPE IL-8 and MCP-1 production. This is the reason why GHSA was used to stimulate MCP-1 and IL-8 secrection from ARPE-19 cells in this study rather than AGE.

The results of this study show that minocycline suppresses glycated albumin induced MCP-1 and IL-8 production from ARPE-19 cells. During autoimmune uveitis, RPE cells are activated and participate in recruitment and stimulation of inflammatory cells into the eye. [267] In vitro, RPE cells have been shown to produce different chemokines such as interleukin 8.[268] It has been extensively described that cytokines such as TNF-α and IL-1β strongly activate RPE cells to secrete various inflammatory mediators including IL-8. [268] It is likely that other stimuli play a role triggering RPE cell activation.

In AMD, macrophages and lymphocytes as well as reactive, migrating or proliferating hRPE cells are often found adjacent to newly formed vessels in the sub-retinal space. [269, 270]

IL-8 has been shown to induce angiogenesis and is present in the vitreous of patients with retinal neovascularisation.[271] MCP-1 involvement in neovascularisation of the posterior segment of the eye has been suggested by several studies. High levels of MCP-1 in vitreous have been detected in proliferative diabetic retinopathy (PDR).[272] Proliferation of RPE cells is

associated with upregulation of MCP-1 expression. [273] Intravitreal injections of anti MCP-1 significantly reduce ischaemia induced retinal neovascularisation.[274]

Human RPE cells respond to monocytes binding to their cell surfaces by secreting IL-8 and MCP-1.[266] Bian et al [266] suggest that MAPK activation and nuclear translocation of NF-κB may represent the major pathway for GHSA signalling in hRPE cells. However, the signal cascades leading to activation of IL-8 and MCP-1 expression remain complicated. Extracellular signal regulated kinase ERK1/2 and p38 mitogen activated protein kinase (MAPK) pathways, and NF-κB induced kinase (NIK) are the major signalling pathways.

MCP-1 belongs to the C-C chemokine family which are named as such because of two adjacent cysteines (amino acids) near their amino terminus. MCP-1 functions as a chemoattractant and as an activator for lymphocytes and monocytes and causes monocyte/macrophage infiltration into tissues. [275] IL-8 is a member of the CXC chemokine family, named as the two terminal cysteines are separated by one amino acid represented in this name by an X. IL-8 is a potent activator and chemoattractant of neutrophils.[276] Transcription factors NF-κB and AP-1 binding motifs are found in MCP-1 and IL-8 gene promoters hence both these chemokines are coinduced in different cells by several stimuli. [276,277]

MCP-1 directed monocyte extravasation and cell-cell contact between monocytes/ macrophages and hRPE cells are important sequential events in retinal diseases, such as proliferative vitreoretinopathy (PVR), AMD and uveitis.

Induced hRPE MCP-1 may initiate and perpetuate ocular inflammation by recruiting and activating monocytes and lymphocytes in diseased retinal tissue.

Bian et al [266] investigated the signal mediators involved in GHSA stimulation of IL-8 and MCP-1 secretion in hRPE cells. hRPE cells were stimulated by GHSA in the presence or absence of a series of kinase inhibitors. Induced IL-8 and MCP-1 mRNA and proteins were determined by reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA). It has been shown [267, 278] that mitogen activated protein kinases (MAPK) plays an important role in AGE signalling. The involvement of extracellular signal regulated protein kinases (ERK) 1/2 was investigated by Bian et al. [266] Two subclasses of MAP kinases are associated with cellular stress response:

1. p38 high osmolarity glycerol (HOG) kinase and c-Jun N-terminal kinase (JNK)/ stress activated MAP kinase (SAPK)

2. Janus kinase (jak) signal transducers and activation of the transcription (STAT) pathway

Bian et al [266] used specific inhibitors of ERK 1/2, p38 and Jak2 to block GHSA stimulation of IL-8 and MCP-1. Inhibitors were used alone and also in combination. The results suggested that GHSA stimulation of MCP-1 expression is mediated by at least three signalling pathways, ERK 1/2, p38 and jak; this contrasts with predominant mediation of IL-8 expression by ERK 1/2. Activation of p38 is required for both IL-8 and MCP-1 induction by GHSA, p38 activation is more effective for inducing MCP-1 than IL-8. Relative contributions

of p38 to IL-8 expression may be cell type dependent. In monocytes, LPS and osmotic stress induced IL-8 expression have been shown to be entirely mediated by p38. [279, 280] In other cell types p38 is insufficient for maximal stimulation of IL-8 production. [281]

The activation of jak appears to be involved in GHSA induction of MCP-1, but not IL-8. [266] Complete blockade of GHSA stimulated MCP-1 production occurred with simultaneous administration of inhibitors for ERK 1/2, p38, and jak2. However, IL-8 production induced by GHSA was insensitive to the jak2 inhibitor AG490 and was not completely inhibited by blocking ERK 1/2 and p38. This suggests that there may be a jak independent mechanism accounting for remaining production of IL-8 in these cells. These mechanisms may be driven by activation of other transcription factors apart from NF-κB such as AP-1.[282]

NF-κB is one of the transcription factors involved in IL-8 and MCP-1 gene expression. [268, 283]

Activation of NF-κB is required for MCP-1 gene expression in hRPE cells.[284]

Activation of NF-κB occurs in response to a wide variety of stimuli such as IL-1β, TNF-α, LPS and many other stress or injury related factors. [285] NF-κB is a pleiotropic transcription factor that regulates activation of various inflammatory genes. [286]

Bian et al [266] determined the involvement of NF-κB in GHSA stimulated hRPE IL-8 and MCP-1 expression in their study.

Their study[266] described four approaches. First, anti NF-κB was used to stain hRPE monolayers immunohistochemically. In unstimulated cells NF-κB was not detected in the nuclei. This contrasted with nuclear translocation of NF-κB that was evident after hRPE cells were treated with 500µg/ml of GHSA for 3 hours.

Secondly, three inhibitors of of NF-κB were utilised.[266] These were BAY 11-7085, caffeic acid phenethyl ester (CAPE), and parthenolide. These act on components of NF-κB in different ways. BAY 11-7085 inhibits IκBα phosphorylation.[287] CAPE is a potent and specific inhibitor of of NF-κB activation.[288] Parthenolide is one of sesquiterpene lactones obtained from varieties of Mexican Indian plants, and prevents the degradation of IκBα and IκBβ.[284] hRPE cells were preincubated with inhibitors, then challenged with GHSA (500µg/ml) for 24 hours. Supernatants were collected for ELISA for IL-8 and MCP-1. All inhibitors completely blocked hRPE IL-8 and MCP-1.

Thirdly, Western blot analysis showed the p65 subunit of NF-κB appeared in nuclear extracts after 3 hours exposure to GHSA (500µg/ml), yet was undetectable in nuclear extracts from unstimulated cells. [266]

The fourth approach described by Bian et al [266] measured activation of NF-κB using an electrophoretic mobility shift assay. Binding of specific probes to the NF-κB binding site of the promoter region in the IL-8 gene was observed 3 hours after induction with GHSA (500µg/ml).

Curcumin is a naturally occurring yellow pigment, isolated from the rhizomes of the plant Curcuma longa (Linn), that is commonly used in Asian cooking as a

flavouring and colouring agent.[289] It has been used in Oriental and Ayurvedic medicine since ancient times. [289] Curcumin has been demonstrated to block a signal leading to activation of NF-κB inducing kinase (NIK). [290, 291] Curcumin was shown by Bian et al [266] to reduce production of IL-8 and MCP-1 in a dose dependent manner.

The phosphatidyl-inositol-3-OH-kinase (P13K)/Akt pathway and the transcription factor AP-1 have been shown to be involved in MCP-1 [291-293] and IL-8 [294-296] expression in cell types other than RPE cells, whereas P13K also activates the NIK pathway. [297]

To investigate differential involvement of phosphoinositide 3 kinase/ Akt in human RPE MCP-1 and IL-8 expression, researchers stimulated hRPE cells with IL-1β and TNF-α by co-culturing with monocytes in the presence or absence of a series of kinase inhibitors. [298] MCP-1 and IL-8 mRNA and protein production were determined by RT-PCR and ELISA.

PI3K comprises a family of lipid signalling enzymes that promote phosphorylation of phosphoinositides. Among them are second messengers that bind to Akt, (also known as protein kinase B, PKB). IL-1, TNF and cell to cell contact have been shown to activate the P13K pathway. [291,294, 299] The specific molecular inhibitor of P13K has been identified as Ly294002. [300] Bian et al [301] demonstrated the important role of the P13K/Akt pathway in the expression of hRPE MCP-1. However, hRPE IL-8 expresion induced by the same stimuli was completely insensitive to Ly294002.[302] This contrasted with

concominant inhibition of both MCP-1 and IL-8 expression by blocking NIK and MAPK pathways.[302] Selective stimulation of either IL-8 or MCP-1 by a given stimulus has been reported in various cell types, however, selective induction of MCP-1, but not IL-8, by a particular signalling pathway is rare. This is similar to observations that GHSA induced MCP-1, but not IL-8 was susceptible to inhibition by AE490, a selective inhibitor of jak2.[266] Ly294002 sensitive P13K is thus a second signalling pathway observed in hRPE cells that selectively stimulates MCP-1. Stimuli may specifically trigger downstream effectors regulating MCP-1 gene expression.

Joint stimulation of IL-8 and MCP-1 mRNA expression is consistent with the finding that both IL-8 and MCP-1 promoters contain common sites for transcription factors NF-κB and AP-1, both of which may be activated by IL-1 and TNF.[298]

NF-κB functions as a dimer, composed of a family of subunits including NF-κB1 (p50), NF-κB2 (p52), c-REL, REL-A (p65) and REL-B .[296] The transcription factor AP-1 is a homo or heterodimer formed by c-Jun, Jun-D, Jun-B, ATF-2, c-Fos, FRA-1, FRA-2 and other members of this family. [296]

Molecular studies of MCP-1 and IL-8 promoters reveal differences between the two genes. In the IL-8 promoter, there is a single NF-κB binding site with high affinity for p65, whereas two NF-κB binding sites A1 and A2 have been found in the human MCP-1 promoter. The A2 site, which has high affinity for c-Rel/p65 is the most important NF-κB binding site for MCP-1 expression.[303] The different

structure and properties of NF-κB sites in the promoters of the two chemokine genes suggests that selective inhibition of MCP-1 expression may result from blocking one of two NF-κB sites through a Ly294002 sensitive P13K pathway. In addition, another mechanism accounting for the selective inhibition of MCP-1 expression by PIK3 inhibitors is that the P13K pathway may activate certain transcription factors required for MCP-1.

The P13K pathway in hRPE MCP-1 induction, but not IL-8, provides putative targets for the selective regulation of hRPE chemokines.

3.9.81 Nucleotide biochemical pathways

Stimuli may arise from pathogens but also from stressed or necrotic cells, and it is now accepted that inflammatory cells respond to molecules normally found inside cells. [304] Among these molecules, nucleotides have been shown to be involved in immune regulation.[305] Nucleotides are released into extracellular fluid through cell lysis, exocytosis of nucleotide concentrating granules, vesicular trafficking or membrane transport proteins. [306] Extracellular nucleotides have autocrine and paracrine roles by activating two families of receptors: P2X receptors, which are ATP gated ion channels and P2Y receptors, coupled to G proteins.[306] $P2Y_2$ receptors have been identified in RPE cells and their activation has been shown to stimulate fluid reabsorbtion and retinal reattachment in a rat model of retinal detachment. [307,308] The presence of P2Y subtypes $P2Y_1$ and $P2Y_6$ has also been demonstrated. [309]

Relvas et al [304] cultured ARPE-19 cells with $P2Y_2$ and $P2Y_6$ receptor ligands ATPγS, UTP, and UDP and also TNF. IL-8 gene transcription and protein production were measured by semiquantative RT-PCR and ELISA. Western blot analysis and RT-PCR were used to investigate ERK 1/2 activation and P2Y expression. Changes in intracellular calcium and cAMP concentration were analysed by spectrofluorometry and radioimmunoassay.

The results demonstrated that ATPγS, UTP, and UDP stimulated both basal and TNF-α induced IL-8 secretion in RPE cells through an ERK 1/2 dependent pathway and that these effects were mediated by $P2Y_2$ and $P2Y_6$ receptors. IL-8 attracts and activates neutrophils.[298] Although the main stimuli of IL-8 secretion in vitro has been TNF-α and IL-1β, stimulation of Toll like receptors 3 and 9 has also been found to induce IL-8 secretion by RPE cells. [310, 311] Toll like receptors recognise pathogen associated patterns and hence “danger signals”. As nucleotides are normally found within cells and are released during tissue damage, extracelluar nucleotides are also considered danger signals. Therefore, RPE cells may act as sentinels that can be alerted when the eye is responding to the threat of pathogen associated infection or inflammatory stress. As there is basal secretion of IL-8 by RPE cells, nucleotide release may help to recruit inflammatory cells from the choroid to the retina. [312] This is an advantage during infection to clear the retina from pathogens. However, if this response becomes dysregulated the eye can be exposed to abnormal IL-8 production and may be involved in the pathophysiology of inflammatory retinal disease. The specific inhibition of MAPK kinase by PD98059 abrogates IL-8

secretion induced by ATPγS, UTP, and UDP in RPE cells. [304] All tested nucleotides induced a strong activation of ERK 1/2. Results suggest that these effects are mediated by $P2Y_2$ and $P2Y_6$ receptors. [304] This suggests that the mechanisms for IL-8 production in RPE cells are multiple and complex and minocycline may not fully suppress GHSA induced IL-8 production due to effects of other biochemical pathways.

3.9.82 Hypoxic inflammation in AMD

The aetiology of AMD is complex and multifactorial. The local regulatory mechanisms of hypoxia, ROS, inflammation and interactions with environmental and life style factors leading to the development of AMD are not yet clearly understood. The hypoxic conditions during inflammation in the retina may be the result of either increased consumption of oxygen due to the increased metabolic activity of the inflamed retina or due to the poor perfusion in the central macula as a consequence of vessel stenosis and microthrombosis[197, 198]

This pathological state may elicit drusen formation, increase oxidative stress and inflammation and lead to the degeneration of RPE cells and progression of AMD.

Cellular damage by oxygen free radicals is the primary driving force for ageing and the expression of transcription factors such as NF-κB that regulate the expression of proinflammatory molecules. The NF-κB protein complex is a critical regulatory mechanism that controls a variety of external and internal danger signals including oxidative stress and inflammation.[232] In its inactive

state the NF-κB complex is retained in the cytoplasm with an IκB protein. In the presence of a stress stimulus, IκB becomes degraded in the ubiquitin-proteasome system, the released NF-κB complex translocates to the nucleus and binds to the promoter area of the target genes that are mostly involved in the regulation of inflammation during ageing. [232]

The elevated levels of ROS strongly linked to AMD pathology are able to activate NF-κB signalling. [313, 314] A binding site for NF-κB has been identified within the HIF-1α promoter. [315] Hypoxia has been shown to to result in activation of NF-κB which can bind to the HIF-1α promoter. Overexpression of the NF-κB complex proteins p50 and p65 may increase HIF-1α promoter activity. [316] Mutation of the NF-κB site in the HIF-1α promoter at -197/-188 base pairs prevents hypoxic induction of HIF-1α promoter activity.[317]This indicates that NF-κB binding to the HIF-1α promoter is a prerequisite for transcriptional activation of HIF-1α by hypoxia.

Hypoxia can also activate the P13K/Akt pathway subsequently leading to the simultaneous activation of the transcription of both NF-κB and HIF-1α genes.[317] ERK 1/2 does not seem to be involved in the NF-κB induced transcriptional activation of the HIF-1α gene. However, ERK 1/2 may be involved in HIF-1α protein stability and/or transactivation without the need for NF-κB activation. It appears that hypoxia leads to activation of NF-κB through a P13K dependent pathway. NF-κB binds to the HIF-1α promoter resulting in an increase in transcription and translation of HIF-1α.[317]

The pathogenesis of AMD is thought to involve reduced oxygen availability together with a chronic low grade inflammatory response. During the pathological ageing process, homeostatic mechanisms may fail in RPE cells. The P13K pathway in conjunction with other members of the NF-κB complex which may modulate HIF-1α target genes may represent a new therapeutic strategy.[317]

Kumar et al [318] cultured human retinal progenitor cells (HRPC) and human umbilical vein endothelial cells (HUVEC) in monolayers of each cell type maintained alone and also co-cultured with the two cell lines. The aim of their study[318] was to elucidate the mechanism of action between the two cell lines that modulates a neovascularisation response. Culture and co-culture was carried out under normoxia (control) or hypoxic (1% O_2) conditions for 24 hours. The secretion of VEGF was examined by ELISA and the expression of VEGF, VEGFR-2, NF-κB and HIF-1α by PCR and Western blotting. The cellular localization of NF-κB and HIF-1α were studied by immunofluorescence and Western blotting. The results [318] showed that hypoxia increased exogenous VEGF expression by 4 fold in HRPC cells with a further 2 fold increase when HRPC was co-cultured with HUVEC. Hypoxia resulted in an increase in NF-κB and HIF-1α, there was a 4 and 6 fold increase during co-culture. Hypoxia induced nuclear translocation of NF-κB in HRPC when exposed to HUVEC hypoxic conditioned media. As nuclear translocation of NF-κB is considered equivalent to activation the findings [318] suggest that direct or indirect communication between HRPC and HUVEC cells results in expression and

activation of transcription factors associated with enhanced expression of pro-angiogenic factors under hypoxic conditions.

Udono et al [262] investigated the effects of hypoxia on the production and secretion of adrenomedullin and endothelin in human retinal pigment epithelial cells. Adrenomedullin (ADM) is a vasorelaxant peptide originally identified from human phaeochromocyctoma. Hypoxia significantly decreased the numbers of ARPE-19 cells and this effect was ameliorated with ADM. [262]

Significant increases in the index of apoptosis were observed in ARPE-19 cells cultured under hypoxic conditions.[262] ADM did not affect the apoptosis rate of ARPE-19 cells exposed to hypoxia. This suggests that the effects of ADM on the cell number of RPE cells under hypoxic conditions are independent of antiapoptotic actions.

3.9.83 Mechanisms for minocyline induced suppresion of IL-8 and MCP-1 production

Pang et al [319] studied the effects of minocycline on LPS stimulated human circulating monocytes in culture. Incubation of primary human monocytes with LPS increased p38 MAPK phosphorylation and this effect was significantly reduced by minocycline in a concentration dependent manner. [319] The effects of minocycline were mediated, at least, in part, by inhibition of NF-κB and p38 activation, the later being completely dependent on the stimulation of the P13K/Akt pathway. This indicates that the effect of minocycline on p38 MAPK activity is exclusively dependent on P13K/Akt signalling. Therefore, minocycline activation of the P13K/Akt pathway plays a significant role in its anti-

inflammatory effects. Minocycline substantially reduced LPS induced NF-κB activation, demonstrating that this pathway is an important target for its anti-inflammatory effects. [319]

The anti-inflammatory effects of minocycline were apparent at a low concentration of 10μM used in the study by Pang et al. [319] This is in the same range of the peak plasma concentrations of 6mg/l (equivalent to 13μM) achieved after a single oral administration of 100 to 200mg of minocycline in humans. [320] However, the plasma concentrations may not accurately reflect minocycline concentrations reaching the retina after crossing the blood retinal barrier.

The studies described above [318, 319] suggest that during hypoxia there may be overexpression of the p65 subunit of the NF-κB transcription factor which may lead to activation of hypoxia inducible factor. Evidence for minocycline suppressing p65 phosphorylation and nuclear translocation of NF-κB accompanied by downregulation of NF-κB activity has been shown by a recent study [321] in ovarian carcinoma cells.

The biochemical inhibitors that were used in the study presented in this thesis were chosen as they specifically inhibited pathways or components of pathways considered to be important mediators of minocycline's action. These were Ly294002 a specific inhibitor of the P13K pathway, SB202190, a p38 inhibitor and JSH-23, an inhibitor of the p65 subunit of NF-κB. As described above, these pathways are interconnected and complex. The mechanisms for glycated

albumin induced MCP-1 production appear to involve the p38 and P13K pathways to a greater extent than the pathways for IL-8 production. Minocycline appears to exert its anti-inflammatory effects through specific targeting of the P13K pathway and also p38. This explains the results of this study where the effects of minocycline in suppressing glycated albumin induced MCP-1 production were greater than similar experiments involving IL-8 production.

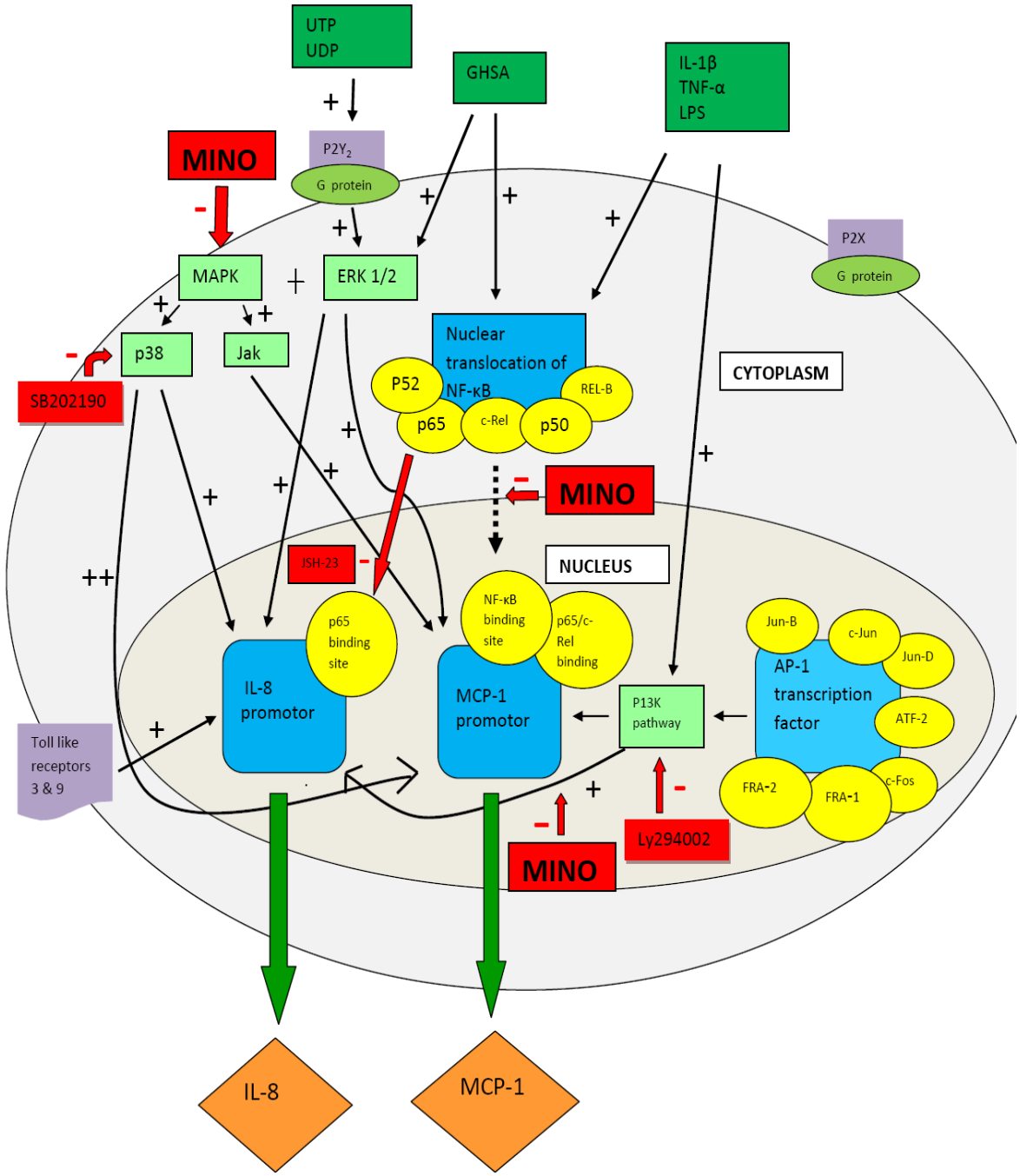

Figure 25: Schematic representation of mechanisms of actions of minocycline, and biochemical inhibitors Ly294002, SB202190 and JSH-23 in suppression of glycated human serum albumin (GHSA) induced IL-8 and MCP-1 production from ARPE-19 cells

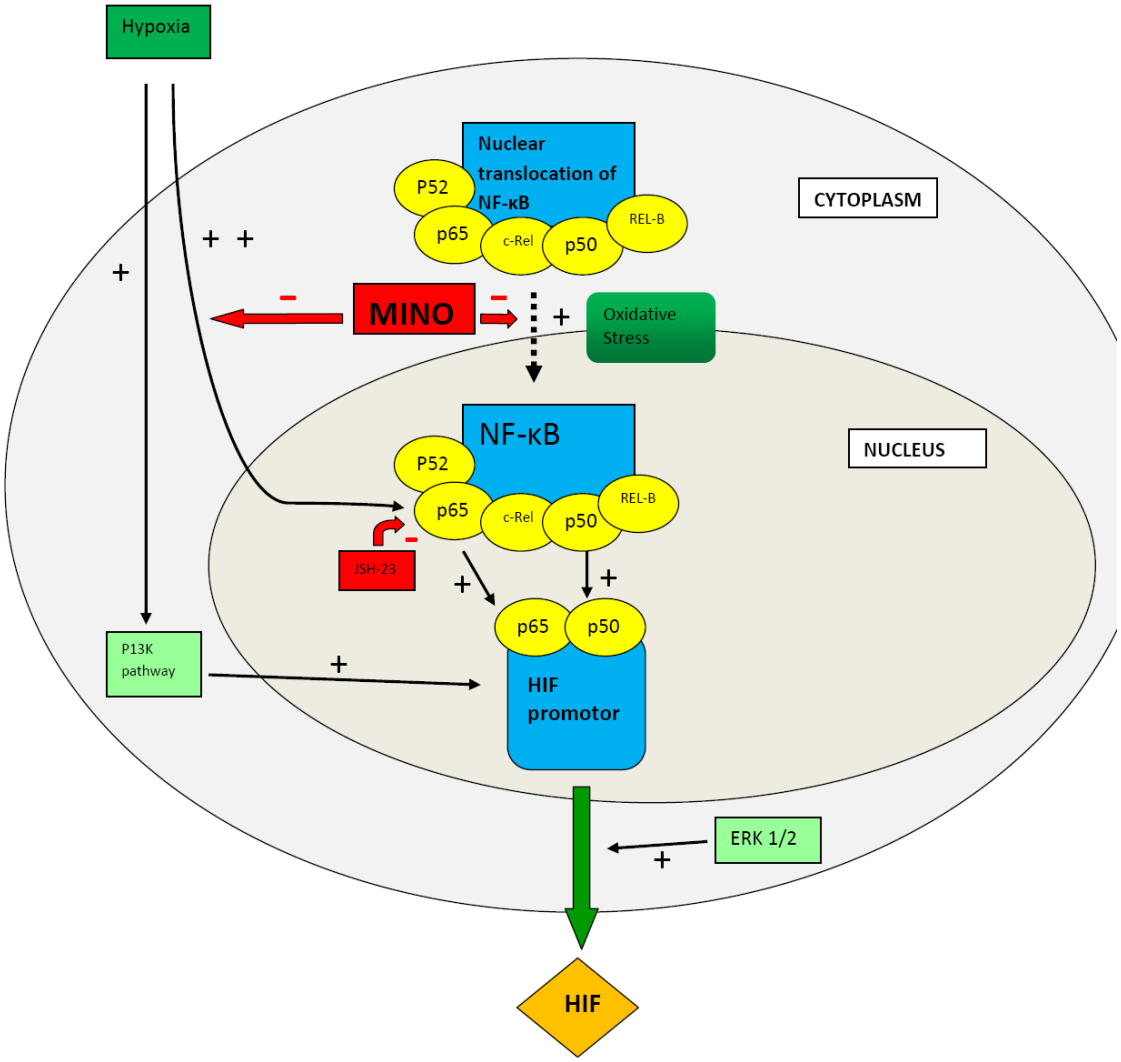

Figure 26: Schematic representation of effects of minocycline and biochemical inhibitor JSH-23 on putative effects of hypoxia and hypoxic inducible factor (HIF) in ARPE-19 cells

3.9.84 Conclusions of preclinical study

The results suggest that minocycline inhibits glycated albumin induced MCP-1 and IL-8 production from ARPE-19 cells in vitro. Minocycline also inhibits MCP-1 and IL-8 production to a lesser extent in hypoxia. The aetiology of neovascular AMD is recognised to be complex and both inflammation and hypoxia contribute to the pathological process culminating in

neovascularisation. This study supports a therapeutic role for minocycline in suppressing IL-8 and MCP-1 production arising from inflammatory and hypoxic pathological processes occurring in neovascular AMD.

Chapter 4

4.0 Clinical Study

4.1 Introduction

The exudative (wet) type of AMD is characterised by the presence of choroidal neovascularisation (CNV).[19] The pathogenesis of neovascular AMD is widely regarded to be complex, involving several intricately linked pathways including oxidative stress, inflammation, immune mechanism and angiogenesis. Inhibition of vascular endothelial growth factor is now in widespread use for treating exudative AMD. Combination therapy in exudative AMD is aimed at targeting the different pathways in the disease process whilst reducing or counteracting the adverse effects of individual therapeutic agents.

4.2 Aims and rationale

The aims were to assess the safety and efficacy of the combined treatment of reduced fluence verteporfin photodynamic therapy (PDT), intravitreal ranibizumab, intravitreal dexamethasone and oral minocycline for choroidal neovascularisation (CNV) secondary to age related macular degeneration (AMD). The hypothesis was that by combining ranibizumab with other CNV modulating agents, the need for ranibizumab retreatment could be reduced while remaining both efficacious and safe.

4.3 Ethics and regulatory approval

The trial was conducted in compliance with the principles of the Declaration of Helsinki (1996), the Principles of Good Clinical Practice, and all of the applicable regulatory requirements. King's College hospital NHS Research Ethics Committee reviewed and approved this research trial.

4.4 Quality Assurance and data handling

Copies of protocols, case report forms, physiological test results, correspondence, informed consents, and other documents relevant to the study were kept on file by the Principal Investigator and archived after completion of the study. The results of the study have been disseminated and presented at scientific conferences and published. [248]

4.5 Methods

4.5.1 Trial design

This was a prospective open label interventional clinical trial of eyes with recent onset CNV secondary to AMD. Patients had to be willing to given written informed consent, undertake the necessary medical tests and treatments and be willing to be followed up. Patients with active medical conditions or a history of a medical condition that would be likely to preclude scheduled study visits such as unstable angina, dialysis and active cancer were excluded. Also, patients who were unable to sign or understand the consent process were excluded.

4.5.2 Eligibility criteria

The inclusion criteria were (1) age ≥ 50 years, (2) clinical diagnosis of wet AMD (3) any active subfoveal CNV (classic and occult) and (4) logMAR best corrected visual acuity (BCVA) of 24-73 letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) chart. Activity of the CNV was determined by recent decline in vision, presence of subretinal fluid or haemorrhage and leakage on fundus fluorescein angiogram. Only one eye of each patient was included in the study. The main exclusions were (1) eyes with CNV from causes other than AMD (myopia, angioid streaks, choroidal rupture, chorioretinal scarring), (2) previous treatment for CNV (PDT, argon laser, anti-VEGF agents, steroids), (3) open angle glaucoma or risk of developing glaucoma and (4) myopia > -8.00 dioptre. Further exclusion criteria were (5) Inability to have a good quality fluoroscein angiogram taken for example, due to head tremor or media opacity (6) Allergy to fluoroscein or verteporfin (7) Previous treatment for retinal detachment (8) Judged by the examining clinician to be at increased risk of retinal detachment due to weaknesses in the peripheral retina (9) Previous photodynamic therapy or other therapy for CNV such as argon laser treatment (10)Patient has currently parcipated in a clinical trial that utilised an investigational drug or treatment within 30 days prior to enrolment in this study (11) Patient taking anticoagulation therapy such as warfarin, with the exception of aspirin and other anti-platelet therapy (12) Exclusion of women of child bearing potential (13) Exclusion of pregnant or lactating women

4.5.3 Ophthalmic assessment schedule

The logMAR BCVA was measured in both eyes after refraction using the modified ETDRS charts. The BCVA, slitlamp biomicroscopic examination of the anterior segment, intraocular pressure measurement and dilated fundus examination of the posterior pole were perfomed at baseline and monthly visits. Baseline and monthly OCT imaging was carried out on the Stratus OCT (Carl Zeiss Meditec) using the fast macular thickness map protocol. Colour fundus photography and fluorescein angiography were performed at baseline, month 6 and month 12.

Table 4: Study visit protocol (X represents procedures completed at visits, X[1] ranibizumab was administered if retreatment criteria met)

Visit number	Visit 1 Screening and treatment	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10	Visit 11	Visit 12
Study month	0	1	2	3	4	5	6	7	8	9	10	11
Procedure												
Informed Consent	X											
Medical history	X											
BCVA	X	X	X	X	X	X	X	X	X	X	X	X
Ophthalmic examination	X	X	X	X	X	X	X	X	X	X	X	X
Tonometry	X	X	X	X	X	X	X	X	X	X	X	X
OCT	X	X	X	X	X	X	X	X	X	X	X	X
Colour fundus photo	X						X					
FFA	X						X					
BP	X											
Weight	X											
Height	X											
PDT & Visudyne	X											
Minocycline	X											
Dexamethasone	X											
Ranibizumab	X	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]

4.5.4 Treatment schedule at baseline

Reduced fluence PDT was administered at baseline. Verteporfin (6mg/m^2) was administered as a standard 10 minute infusion, and the PDT laser was applied with a reduced fluence (25 J/cm^2, 300mW/cm^2) 5 minutes after the infusion of the drug for 83 seconds. This was followed by a single intravitreal injection of ranibizumab 0.3mg/ 0.05ml and dexamethasone 200µg in 0.05ml that were combined in the same syringe (total volume 0.1ml).

The intravitreal injection was administered using a standard sterile technique. Measurement of intraocular pressure was performed prior to and 30 minutes after the injection. A post injection course of ofloxacin eyedrops 3 times a day for 4 days was prescribed.

4.5.5 Subsequent treatment regimen

All subsequent intravitreal injections of ranibizuma were only administered if they fulfilled the retreatment criteria. Oral minocycline 100mg daily was started the next day after the baseline treatment was administered and continued for 3 months. Patients were instructed to return all used and unused drug packages to ascertain compliance.

4.5.6 Retreatment criteria

Re-injection with ranibizumab was indicated if any of the following were present at the monthly follow up visits:

1. A loss of > 5 letters of refracted logMAR BCVA recorded on the modified ETDRS chart when compared to the previous month
2. An increase in central retinal thickness (CRT) of >100µm measured on a fast macular thickness map when compared to the previous month
3. Fresh macular haemorrhage on dilated fundus biomicroscopy

4.5.7 Outcome measures

The primary efficacy outcome measures were:

1. The mean changes in BCVA from baseline to study completion
2. The proportion of patients with stable vision (a loss or gain of less than 15 letters)
3. The mean ranibizumab retreatment rate per study eye over the follow up period

The secondary outcome measures were:

1. The proportion of study eyes which gained ≥ 5 letters of BCVA
2. The proportion of study eyes which lost ≥15 letters of BCVA
3. Mean final BCVA
4. The mean change in the CRT on OCT

The safety profile was assessed by visual acuity, tonometry, ophthalmic examinations, adverse events and vital signs.

4.5.8 Statistical analysis

The proof of concept safety hypothesis used in other combination studies to measure possible safety implications and the relationship between the concomitant administration of two drugs was used in this study (FOCUS and PROTECT). [322, 323] Enrolment of 19 patients was needed to allow a 95% chance of observing at least 1 adverse event with a true rate of occurrence of ≥4% within the treatment period, based on the 3% rate of moderate visual loss reported in the PROTECT study[322], the study that assessed safety of same day standard PDT and ranibizumab and the study that assessed outcomes of combined bevacizumab and dexamethasone with PDT that showed 3.8% lost 3 or more lines. [324] These safety assessments included the incidence of severe vision loss (number of patients losing 30 letters of BCVA from baseline) and the incidence of moderate vision loss (number of patients losing 15 letters of BCVA from baseline).

4.6 Results

Nineteen eyes of 19 patients were recruited into the study. One patient was withdrawn from the study as a result of epidural haematoma after a retreatment at month 2. Three patients were retreated with 0.5mg/ 0.05ml of commercially available ranibizumab (2 from visit 10 and 1 from visit 8) due to non availability of the study specific ranibizumab (0.3mg/0.05ml) preparation. The baseline characteristics are described in table 5.

Table 5: The baseline characteristics of the study cohort

Baseline characteristics	Number of eyes (total n = 18)
Mean age, years	79.2±6
Laterality (OD:OS)	11:7
CNV type	
Predominantly classic	3 (15.7%)
Minimally classic	3 (15.7%)
Occult not classic	12 (63.2%)
Retinal angiomatous proliferation	1 (5.3%)
Mean baseline visual acuity (ETDRS)	58±11.4
Mean baseline CRT (microns)	315.1±65.4
Mean angiographic lesion size mm^2	6.75±2.78 (range 1.9 to 10.9)

4.6.1 Visual acuity outcomes

The mean change in BCVA at the final follow up was -5.0 (± 10.5) ETDRS letters (figure 27). The final mean BCVA was 52.6 ± 16 ETDRS letters (range 7-76). Eighty nine percent (16/18 eyes) had stable vision losing fewer than 15 ETDRS letters. None of the study eyes gained more than 15 ETDRS letters, although 3/15 eyes gained more than 5 ETDRS letter (7, 11 and 12 letter gain). One eye suffered a moderate visual loss of 24 letters, and another eye had severe visual loss of 30 letters.

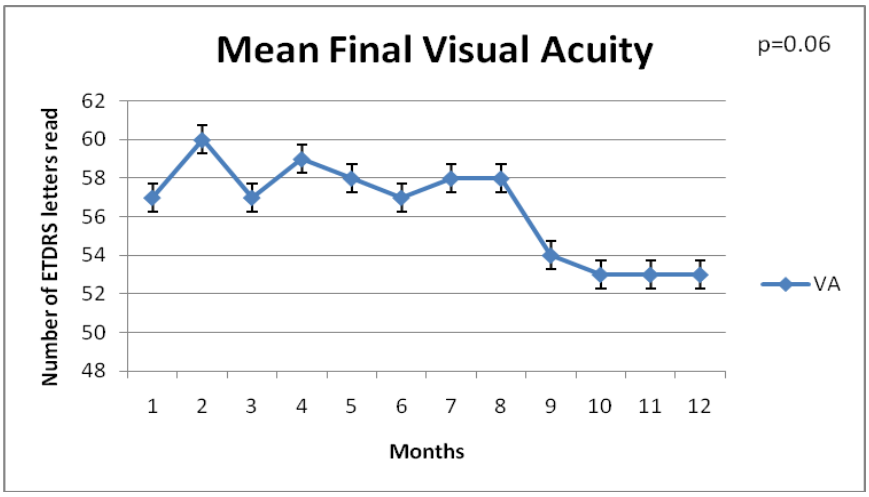

Figure 27: Mean final visual outcome at the final follow up at 12 months. The final mean BCVA was 52.6± 16 ETDRS letters. Mean change in BCVA was -5.0 (±10.5) ETDRS letters. This was not significant (p = 0.06).

4.6.2 OCT outcomes

The mean reduction in CRT was 66.3 (±75) µm. The mean final CRT on OCT was 248 ± 52µm. The greatest reduction in CRT measurements occurred 1 month after the combined treatment was administered after which it remained relatively stable (figure 28).

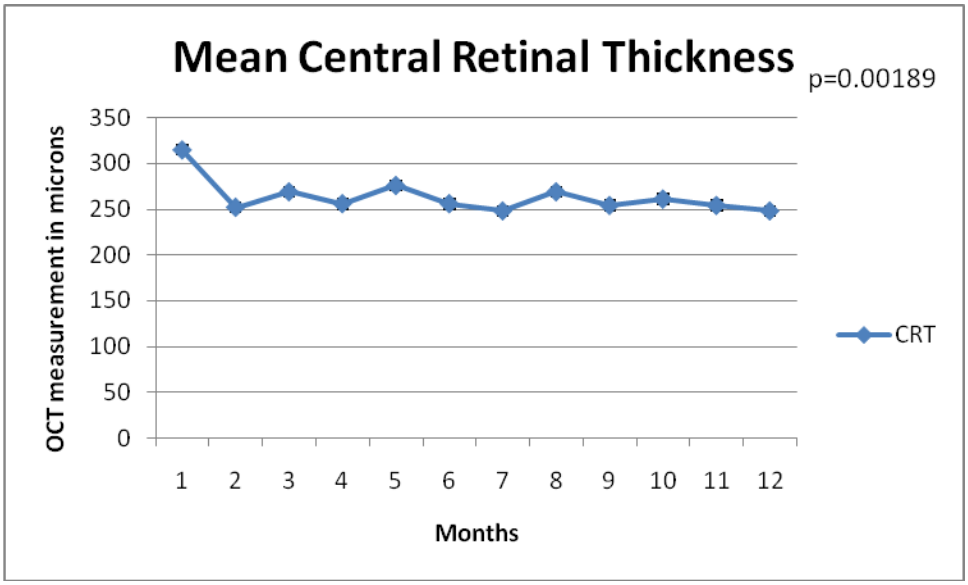

Figure 28: Mean change in central retinal thickness (CRT) during the study period. The mean final CRT on OCT was 248 ± 52µm. The mean reduction in CRT was 66.3 ±75µm. The greatest reduction in CRT measurements occurred 1 month after the combined treatment was administered after which it remained relatively stable

4.6.3 Retreatment outcomes

The mean total number of ranibizumab injections given was 3.4 (baseline ranibizumab + ranibizumab retreatments). The average number of months from baseline treatment with combined reduced fluence verteporfin PDT, ranibizumab, dexamethasone and minocycline to retreatment with ranibizumab monotherapy was 2.6 months (range 1-6). Two eyes required 5 ranibizumab retreatments, and the mean changes in visual acuity in these eyes were -12 and -6 ETDRS letters, respectively. Three eyes required only 1 additional ranibizumab retreatment, while the rest of the cohort needed 2-3 injections.

4.6.4 Safety assessment

One eye suffered a moderate visual loss of 24 letters. This 73 year old patient had a predominantly classic lesion with a pretreatment baseline visual acuity of 31 letters, CRT of 319µm and lesion size of 9.08mm^2. In the first month following baseline combination treatment, the vision improved to 37 letters and CRT was reduced to 182µm. Two additional ranibizumab retreatments were given at follow up months 3 and 5. However, the visual acuity dropped by 16 letters compared to the previous month and continued to progressively deteriorate. The final visual acuity was 7 letters and CRT was 267µm. The visual loss was attributed to central subretinal fibrosis, and the final size of the fibrotic lesion was 14.502mm^2.

Severe visual loss of 30 letters was reported in 1 eye. This 71 year old patient had an occult lesion with a baseline visual acuity of 62 letters, CRT of 279µm and lesion size of 9.266mm^2. Baseline combination therapy and 1 ranibizumab retreatment at follow up month 3 stabilised the vision for 5 months. Despite 2 further ranibizumab injections, the vision progressively deteriorated from month 6 to a final visual acuity of 32 letters. The final size of the fibrotic lesion was 16.037mm^2.

None of the patients had any inflammatory response, raised intraocular pressure or cataract extraction during the study period. There was 1 case of mild gastrointestinal disturbance secondary to oral minocycline, but the patient was able to complete the 3 month course. There was 1 study related death. This 89 year old patient had received both the combination treatment regime at

baseline and a retreatment with ranibizumab at month 2. She then died of an epidural haematoma a week following the treatment. This was reported as a suspected unexpected serious adverse reaction (Appendix F, page 225).

4.7 Discussion

This pilot study was powered to determine the adverse events when a quadruple therapy of reduced fluence PDT, intravitreal ranibizumab and dexamethasone and oral minocycline are used as a treatment option for neovascular AMD. The efficacy of individual components of the combination therapy was not evaluated in this study.

The study showed that quadruple therapy can be delivered safely. Reduced fluence PDT with 25 J/cm^2 reduces the choroidal hypoperfusion, inflammation, vascular leakage and VEGF upregulation that is associated with standard fluence PDT. [322-325] It was used only once during the course of treatment in combination with ranibizumab and dexamethasone. Dexamethasone provided additional anti-inflammatory, antifibrotic, anti-proliferative and anti-VEGF effects. It is more rapidly cleared from the vitreous that triamcinolone with a lower risk of raised intraocular pressure and cataract formation. [246, 247] Oral minocycline, a semi synthetic tetracycline derivative, was used for its immunomodulatory, anti-angiogenic, antimetalloproteinase, anti-oxidant and anti-apoptotic effects. [254] A reduced dose of ranibizumab (0.3mg in 0.05ml) was used, given the combined angio-occlusive effect of PDT and the anti-angiogenic effects of dexamethasone and minocycline. No angiographic evidence of choroidal non perfusion was noted at 6 months in any of the patients in this case series, unlike other

combination studies. [326] This may be because choroidal perfusion defects are noted within a week of combination treatment while this study's protocol was monthly visits.

Two of the 18 patients (11%) lost more than 3 lines of vision due to subretinal fibrosis. In contrast, the PROTECT study [322] showed that only 1/32 lost more than than 15 letters at 9 months. The difference between the two studies was that intravitreal ranibizumab was given monthly for 4 months in the induction phase and PDT was administered at 12 weekly intervals if angiographic leakage was present at 3, 6 and 9 months in the PROTECT study [322] compared to an 'as needed' strategy for re-injection with ranibizumab and no further PDT in this study.

Both patients in this study lost the vision due to subretinal fibrosis, and the increase in fibrosis was noted at the 5th and 8th months, respectively. The second injection of intravitreal ranibizumab was only given at 3 months in both cases, and the change in vision at that point was 0 and +2 and re-injection of ranibizumab was given for recurrence of fluid. This suggests that in combination treatment, retreatment with intravitreal ranibizumab based on recurrent fluid is an insufficient retreatment criterion. This trial was designed before OCT guided dosing schedules were published. Clearly, this study shows that it is insufficient to treat recurrent fluid only.

This study did not show the initial steep gradient of gain in vision noted in the PROECT study [322] and the ranibizumab monotherapy trials (MARINA[104] and

ANCHOR [105]), suggesting that the quadruple therapy at baseline (or only 1 intravitreal ranibizumab injection) is insufficient to improve vision. Repeated injections of ranibizumab in the induction phase are required to achieve an improvement in vision.

The study results correlate well with the observation that reduced levels of aqueous VEGF and pigment epithelium derived factor are associated with ranibizumab therapy regardless of combined therapy with PDT. The reduction levels of VEGF and pigment epithelium derived factor also correlated with anatomical improvements in the macula, suggesting that ranibizumab is the main driver of outcomes. [327]

This study mirrors the outcomes of other multicentre trials on combination treatments of ranibizumab and PDT such as the FOCUS study. [323]

The MONT BLANC study [328] that evaluated the superiority of standard PDT with ranibizumab compared to ranibizumab monotherapy also showed that an induction of 3 injections of intravitreal ranibizumab is required for an improvement of vision (+7.1 ETDRS letters). The study also showed that a PRN schedule in the maintenance phase of either ranibizumab monotherapy or the combination of standard PDT with ranibizumab booster is insufficient to sustain the improvement of vision. [328]

This study reiterates that aggressive retreatment strategies have to be initiated to improve and sustain the effect. [329] Although ranibizumab monotherapy is a burden to patient, treating physicians and health care systems, it is the only

treatment that can sustain an improved vision obtained at induction if strict re-treatment criteria are implemented.

Chapter 5

5.0 Current Treatment Approaches for neovascular AMD

Following the results of pivotal clinical trials (MARINA[104] and ANCHOR[105]) published in 2006, and NICE approval of Ranibizumab in 2007 and Aflibercept in 2013 current standard NHS treatment for neovascular age related macular degeneration involves intravitreal injections of ranibizumab or afilbercept at regular intervals. Various studies have been published (PIER[113], PRONTO[117]) in an attempt to demonstrate vision can be maintained with a less intensive injection regimen. The results of the VIEW studies [330] with aflibercept offered a direct comparision between 2 monthly aflibercept and monthly ranibizumab and showed equivalent visual gains. However, the VIEW study did not include a comparision group of two monthly ranibizumab. [330]

5.1 Radiation therapy

The underlying pathological mechanisms in neovascular age related macular degeneration have been postulated to be similar to those of a proliferative wound healing response.[331] Radiation therapy targets proliferating cells in neovascular age related macular degeneration including fibroblastic, inflammatory and endothelial cells.[331]

Radiation therapy for medical use is usually divided into two main categories depending on its method of delivery to the tissue. Brachytherapy uses a radiation source which produces ionising radiation as it decays and emits

energy. The radiation source is delivered directly to the lesion by surgery. External beam therapy is delivered from an external source in which radiation is formed into beams which can be projected at an internal body tissue. [331]

Two main approaches to radiation therapy in neovascular AMD are under investigation, epimacular brachytherapy (VIDION; NeoVista Inc. Fremont,CA) and stereotactic radiosurgery (IRay system; Oraya Therapeutics Inc Newark,CA)[331]

The beta radiation (approximately 24 gray delivered to the macular lesion) in epimacular brachytherapy is delivered via a probe through a pars plana vitrectomy. Potential risks of intraocular radiation include retinopathy, optic neuropathy and cataract. [331]However, the dose delivered to nearby structures is reported to be below the safety threshold to cause damage. Vitrectomy is postulated to improve the oxygenation of the inner retina via diffusion from the aqueous humour.[332] Reduced oxygen tension within the inner retina may instigate CNV formation. The MERITAGE study[333] was a multicentre study to evaluate the safety and efficacy of focal delivery of radiation in patients that require persistent injections of anti VEGF therapy. Fifty three eyes of fifty three participants with neovascular AMD were included. Patients underwent pars plana vitrectomy with a single dose of 24Gy of epimacular brachytherapy delivered via an intraocular handheld cannula containing a strontium 90/ yttrium 90 source positioned over the active lesion. Retreatment with ranibizumab was administered monthly as needed, using predefined retreatment criteria. Optical coherence tomography (OCT) was undertaken monthly. Primary outcome

measures at 12 months were proportion of patients with stable vision (losing < 15 letters on the ETDRS chart), and mean number of anti-VEGF injections. Prior to commencement of the trial patients had received a mean number of 12.5 anti-VEGF injections. After a single treatment with epimacular brachytherapy, 81% maintained stable vision, the mean number of anti-VEGF retreatments was 3.49 in 12 months. Mean change in vision was -4.0 ETDRS letters with a standard deviation of ±15 letters. Cataract was documented in 16 (30%) patients. The conclusions were that EMB produced stable vision in most patients with previously treated active disease and may have a role to decrease the need for frequent anti-VEGF retreatment.[333]

CABERNET[334] was a multicentre randomised controlled trial evaluating the safety and efficacy of epimacular brachytherapy in treatment naive patients. 494 patients with treatment naive classic, minimally classic, and occult lesions with neovascular AMD were randomised 2:1 into EMB or ranibizumab control arm. The EMB arm received two mandatory monthly loading injections of 0.5mg of ranibizumab. The control arm received three mandatory monthly loading injections of ranibizumab then quarterly injections. Both arms received monthly as needed (PRN) retreatment. The main outcome measures at 24 months were the proportion of patients losing or gaining 15 letters of vision on the ETDRS chart from baseline vision.[334]

Results[334] demonstrated that 77% of the EMB arm and 90% of the control arm lost fewer than 15 letters. This difference did not meet the prespecified 10% non inferiority margin. EMB did not meet the superiority end point for the proportion

of patients gaining more than 15 letters (16% EMB versus 26% for the control group). The differences were statistically significant for occult lesions (favouring controls) but not for classic and minimally classic lesions. [334] End change in vision was -2.5 letters in the EMB arm and +4.4 letters in the control arm. Patients in the EMB arm received a mean number of 6.2 ranibizumab injections versus 10.4 in the control arm. Postvitrectomy cataract occurred in 54% of the EMB arm versus 18% in the control arm. Mild non vision threatening radiation retinopathy occurred in 3% of the EMB arm. Despite an acceptable safety profile, the conclusions of the trial were that the two year data do not support the routine use of EMB for treatment naive neovascular AMD.[334]

MERLOT is a UK multicentre randomised controlled trial in patients who have already commenced anti-VEGF therapy. The aim is to compare epimacular brachytherapy and as required ranibizumab with ranibizumab monotherapy. This study has an estimated enrollment of 363 patients and results are yet to be published.

Sterotactic radiotherapy is a technique for targeting beams from different angles onto the target area thereby avoiding exposure to surrounding healthy tissue. [331] The IRay system utisilises a low energy X-ray source so avoiding the need for a high degree of radiation shielding. The patient is secured in place with a head restraint with lead backing preventing radiation travelling beyond the patient. A robotically controlled delivery system connects the radiation source to the patient using a contact lens and 25mmHg suction. Two to three beams of radiation are delivered to overlap on the foveal centre through the inferior pars

plana region of the sclera (5, 6 and 7 o'clock), dispersing the scleral entry dose and minimising exposure of the lens and optic nerve. The eye is continually tracked during treatment and an inbuilt safety feature interrupts the radiation treatment if the eye moves out of position. [331]

The safety and efficacy of low voltage external beam stereotactic radiotherapy (SRT) for neovascular age related macular degeneration was studied in a randomised, double masked sham controlled multicentre clinical trial. [335] Two hundred and thirty patients with onset of neovascular AMD within the previous three years who had received three or more injections of ranibizumab or bevacizumab within the preceeding year and needed continual treatment were recruited. Patients were randomised 2:1:2:1 to 16 Gy SRT plus PRN ranibizumab, sham SRT 16 Gy plus PRN ranibizumab, 24 SRT Gy plus PRN ranibizumab and sham SRT 24 Gy plus PRN ranibizumab respectively.[335] The primary efficacy end point was the mean number of ranibizumab injections at week 52. Secondary endpoints were change in mean BCVA, loss or gain of 15 letters of vision on the ETDRS chart, and change in angiographic total lesion size and choroidal neovascularisation (CNV) lesion size. The results[335] demonstrated that both the 16Gy and 24 Gy SRT treatment arms received significantly fewer ranibizumab treatments compared to the sham arms, however, in terms of clinical impact this only equates to an equivalent mean reduction of 1.5 injections per year. Change in mean VA was -0.28, +0.40 and -1.57 letters for the 16 Gy, 24 Gy and sham arms respectively. Across all arms vision was maintained with approximately 90% of patients losing fewer than 15

letters of vision. Ony 4% of patients across all arms gained 15 letters of vision. The numbers of adverse events were similar across all arms and no adverse events were attributed to radiation. The conclusions were that a single dose of SRT reduces ranibizumab retreatment for patients with neovascular AMD and there were no concerns over patient safety effects over a one year period. SRT was associated with relatively well preserved vision over a one year period. [335]

5.2 Combination studies

Research has also been conducted to examine treatment outcomes utilising a combination of PDT and anti-VEGF drugs. The aims were to attempt to reduce the treatment burden of anti VEGF monotherapy whilst maintaining the vision gains achieved with anti-VEGF monotherapy. The SUMMIT clinical trial programme evaluated the efficacy and safety of verteporfin PDT in combination with ranibizumab compared with ranibizumab montherapy in various forms of CNV. This comprised three studies, MONT BLANC [328], DENALI[336] and EVEREST.[337]

Polypoidal choroidal vasculopathy (PCV) is a distinct clinical entitity and regarded as part of the spectrum of neovascular age related macular degeneration. The condition is associated with recurrent macular serous leakage and haemorrhage. It has been shown that this condition occurs more frequently in Asian populations.[338]

The EVEREST trial[337] was part of the SUMMIT program and conducted only in Asia. In the EVEREST trial[337] treatment naive patients with ICG verified PCV

were randomised to verteporfin PDT (6mg/m^2), ranibizumab, or a combination of both. The primary end point was the proportion of patients with ICG assessed complete regression of polyps at 6 months follow up. Results showed that the combination of PDT with ranibizumab or PDT alone was superior to ranibizumab monotherapy in achieving complete polyp regression (77.8 and 71.4 versus 28.6%, $p < 0.01$). Combination treatment also showed superior improvements in vision (+10.9 letters compared to +7.5 letters for verteporfin PDT and +9.2 letters for ranibizumab monotherapy).

As part of the SUMMIT program, the MONT BLANC [328](conducted in Europe) and DENALI[336] (conducted in the USA and Canada) studies evaluated combination therapy in patients with CNV because of neovascular AMD. The DENALI study[336] showed noninferiority of ranibizumab in combination with verteporfin PDT versus monthly ranibizumab monotherapy in patients with subfoveal CNV secondary to AMD.[339] Clinically meaniful visual gains were maintained with fewer intravitreal injections over 12 months. Ranibizumab treatment free intervals of over 3 months were achieved in 92.6% and 83.5% of patients randomised to verteporfin standard fluence or verteporfin reduced fluence respectively associated with a mean of 5.1 and 5.7 ranibizumab injections respectively. Patients in the ranibizumab monotherapy arm received 10.5 injections. [339]

Currently, combination therapy with PDT and anti-VEGF injections is used as second line therapy when patients do not respond to anti-VEGF monotherapy or

when the treatment burden of monthly injections is too great. Combination therapy is used as primary treatment for idiopathic PCV.

Retinal angiomatous proliferation is a subtype of neovascular age related macular degeneration. This condition often requires repeated anti-VEGF injection treatment. Triple therapy involving a combination of verteporfin PDT, intravitreal anti VEGF and intravitreal steroid (dexamethasone or triamcinolone) has been used for these difficult to treat lesions. Small prospective randomised studies [340] of RAP lesions show vision was stabilised in all treatment groups but results were more favourable with a triple therapy regimen.

5.2.1 Antiplatelet derieved growth factor and antivascular endothelial growth factor

PDGF regulates cell growth and division and is an important factor in new blood vessel formation from existing vascular tissue. Several subtypes of PDGF exist as part of the PDGF family, such as PDGF-CC. This factor increases the survival of vascular endothelial cells and promotes the proliferation, survival and migration of fibroblasts and pericytes. Inhibition reduces macrophages infiltration during CNV formation. [341] Both choroidal and retinal vascularisation were shown to be suppressed when inhibition of PDGF-CC occurred in preclinical studies.[342] This provides a rationale for combining anti-PDGF together with anti-VEGF agents to treat CNV. The aim of anti-PDGF treatment would be to strip pericytes from maturing neovascular tissue thus leading to

increased susceptibility to anti-VEGF therapeutic agents. Anti-PDGF treatment in isolation is unlikely to show an effect. [342]

A prospective randomised controlled trial [343] of patients with neovascular AMD was conducted to compare anti-PDGF (Fovista, Ophthotech New York, USA) administered in combination with ranibizumab to monthly ranibizumab. Patients receiving the combination regimen at monthly intervals gained significantly more vision (mean gain +10.6 letters) at 24 weeks compared with patients receiving ranibizumab monotherapy (+6.5 letters, p=0.019). [343]

Future treatment approaches are likely to target an increasing number of growth factors in an attempt to reduce the treatment burden for individual patients and healthcare providers.

5.3 General Conclusions

The results of a pilot trial of combination therapy incorporating minocycline for neovascular age related macular degeneration have shown that this treatment is safe. However, the results were not superior than anti-VEGF monotherapy.

The results of the pre-clinical research on a putative therapeutic role for minocycline in the treatment of neovascular age related macular degeneration have shown that there is a narrow therapeutic range for treatment. Minocycline appears to protect cells from hypoxia, the molecular mechanisms are complex and were not investigated in detail in this study. In this study minocycline was found to inhibit glycated albumin induced MCP-1 and IL-8 production from ARPE-19 cells in culture. The limitations of this study were that this model is

unlikely to accurately represent in vivo pathology in AMD due to the complex interacting molecular mechanisms involved. However, the results do support a therapeutic role for minocycline in the early inflammatory pathological process. Further studies will be required to determine the future therapeutic potential of minocycline in age related macular degeneration.

5.4 Future work

Future studies will be required to elucidate fully the detailed molecular mechanisms through which minocycline acts. Putative targets apart from inflammatory cytokine suppression include cellular tight junction proteins and signalling. A recent study by Abcouwer et al [344] demonstrated that minocycline prevented ischaemia reperfusion induced permeability and tight junction disorganisation in a rat model of retinal neurodegeneration. Similarly, a small clinical study of five patients with diabetic macular oedema found that 6 months of minocycline treatment reduced vascular fluoroscein leakage and diminished mean retinal thickness. [345] This indictates potential future roles for minocycline to prevent the vascular leakage associated with neovascular AMD.

Autophagy is another process in which minocycline may have a future therapeutic role.

During the last decade there has been a greater understanding of the process of autophagy. In normal physiological conditions, autophagy serves as a housekeeping process through which cytoplasmic proteins and damaged cellular organelles, such as dysfunctional mitochondria are removed.[346]

Autophagy plays a key role in cellular homeostasis and this process can be stimulated to cope with excessive organelle damage, aggregate removal, and pathogen defense.[347]

As accumulation of proteins and damaged organelles are generally considered to occur in the aging RPE and in AMD, it is reasonable to postulate that a breakdown in the recycling capacity of autophagy may have a strong association. Starvation has been used as an inducer of autophagy in most studies, however, oxidative stress has also been acknowledged as a regulator of autophagy. [348] Autophagy proteins are strongly expressed in the RPE. [349]

Many of the pathogenic features of RPE in AMD such as lipofuscin accummulation, susceptibility to oxidative stress and lysosomal dysregulation have an association with autophagy. It is currently unclear whether changes in autophagy are a cause or consequence of disease. A recent study [350] utilising cultured RPE cells and AMD donor tissue showed that under conditions of increased mitochondrial damage, autophagy markers were upregulated and that drusen in AMD donor eyes contain markers for autophagy. This suggests that increased autophagy and release of intracellular proteins by the aged RPE may contribute to the formation of drusen. Ischaemia and hypoxia are considered to contribute towards the pathogenesis of neovascular AMD and hypoxia induced autophagy has been demonstrated [351] as a cell survival mechanism. Induction of autophagy can be obtained through inhibition of the mitogen activated protein kinase/ extracellular signal regulated kinase, phosphoinositide 3-kinase-AKT and MTOR signalling pathways. Inhibitors of P13 kinases such as Ly2949002 can inhibit autophagy [352] and this same

pathway has been identified as a putative mechanism for the actions of minocycline. This suggests that minocycline is a plausible therapeutic option to target regulation of autophagy signalling pathways in AMD. However, the complexity of autophagy as a pharmacological therapeutic target in AMD therapy means, to date, there is no consenus as to whether autophagy inhibitors or activators would be beneficial in AMD therapy.

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Appendix A

Detailed protocol for human IL-8 ELISA

A Reconstitution and dilution of Hu IL-8 standard
Glass or plastic tubes may be used for standard dilutions

1. Reconstitute standard to 10.0ng/mL with *Standard Dilutent Buffer.* Refer to standard vial label for instructions. Swirl or mix gently and allow to stand for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.

2. Add 0.100mL of the reconstituted standard to a tube containing 0.9mL *Standard Dilutent Buffer.* Label as 1000pg/mL Hu IL-8. Mix

3. Add 0.300mL of *Standard Dilutent Buffer* to each of 6 tubes labelled 500, 250, 125, 62.5, 31.2 and 15.6pg/mL Hu IL-8.

4. Make serial dilutions of the standard as described in dilution table. Mix thoroughly between steps.

B Dilution of Hu IL-8 Standard

Table 6: Dilution of IL-8 Standard

Standard	Add:	Into:
1000 pg/mL	Prepare as described in step 2	
500 pg/mL	0.300 mL of the 1000 pg/ mL std.	0.300 mL of the Diluent Buffer
250 pg/mL	0.300 mL of the 500 pg/mL std.	0.300 mL of the Diluent Buffer
125 pg/mL	0.300 mL of the 250 pg/mL std.	0.300 mL of the Diluent Buffer
62.5 pg/mL	0.300 mL of the 125 pg/mL std.	0.300 mL of the Diluent Buffer
31.2 pg/mL	0.300mL of the 62.5 pg/mL std.	0.300 mL of the Diluent Buffer
15.6 pg/mL	0.300mL of the 31.2 pg/mL std.	0.300 mL of the Diluent Buffer
0 pg/mL	0.300 mL of the Diluent Buffer	An empty tube

Discard all remaining reconstituted and diluted standards after completing the assay.

Return the *Standard Diluent Buffer* to the refrigerator.

C Storage and final dilution of streptavidin- HRP

1. Dilute 10µl of this 100x concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8 well strip used in the assay. Label as Streptavidin-HRP working solution.

2. **Table 7: Dilution of streptavidin- HRP**

# of 8 well strips	Volume of streptavidin-HRP concentrate	Volume of Diluent (mL)
2	20µl solution	2
4	40µl solution	4
6	60µl solution	6
8	80µl solution	8
10	100µl solution	10
12	120 µl solution	12

2. Return the unused *Streptavidin-HRP* concentrate to the refrigerator.

D Dilution of wash buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionised water (eg. 50mL may be diluted up to 1.25 litres, 100mL may be diluted up to 2.5 litres). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

A standard curve must be run with each assay

1. Determine the number of 8 well strips needed for the assay.

   Insert these in the frame(s) for current use. (Re-bag extra strips and

   frame. Store these in the refrigerator for future use).

2. Add 50µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.

3. Add 50µl of standards, samples or controls to the appropriate microlitre wells

4. Pipette 50µl of biotinylated anti-IL-8 (*Biotin Conjugate)* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.

5. Cover plate with a *plate cover* and incubate for 1 hour and 30 minutes at room temperature.

6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times.

7. Add 100µl Streptavidin- HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in REAGENT PREPARATION AND STORAGE.

8. Cover plate with a *plate cover* and incubate for 30 minutes at room temperature.

9. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times.

10. Add 100µl of *Stabilised Chromogen* to each well. The liquid in the wells will begin to turn blue.

11. Incubate for 30 minutes at room temperature and in the dark. Do not cover with Aluminium foil. The incubation time for the chromogen substrate is often determined by the microtitre plate reader used. Many plate readers have the capacity to record a maximal optical density (O.D.) of 2.0. The O.D values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450nm can only be read after the *stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D. stopping the assay after 20 to 25 minutes is suggested.

12. Add 100µl of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.

13. Read the absorbance of each well at 450nm having blanked the plate reader against a chromogen blank composed of 100µl each of *Stabilised Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution.*

14. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting). Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.

15. Read the Hu IL-8 concentration for unknown samples and controls from the standard curve plotted in step 14. (Samples producing signals greater than that of the highest standard (1000 pg/mL) should be diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor).

Detailed protocol for human MCP-1 ELISA

Preparation of reagents

1. Bring all reagents and samples to room temperature (18-25°C) before use.

2. Sample dilution: 1X assay diluent buffer is used for dilution of cell culture medium dilution

3. Assay diluent was diluted 5 fold with deionised or distilled water before use

4. Preparation of standard: The vial was briefly spun, then 400µl 1X assay diluent was added to prepare a 100ng/ml standard. The powder was dissolved thoroughly by gentle mix. 5µl of this MCP-1 standard was added to a tube containing 995µ 1X assay diluent to prepare a 500pg/ml stock standard solution. 300µl of assay diluent was pipetted into 7 empty tubes. The stock standard solution was used to produce a a dilution series as shown in the table below. Each tube was mixed thoroughly

before the next transfer. Gentle vortex was used to mix each tube. The 1 X assay diluent served as the zero standard (0pg/ml).

Table 8: Dilution of MCP-1 standard

Standard	Add:	Into:
500 pg/mL	Prepare as described in step 2	
500 pg/mL	200µl of the 500 pg/ mL std.	300µl of the Diluent Buffer
200 pg/mL	200µl of the 500 pg/mL std.	300µl of the Diluent Buffer
80 pg/mL	200µl of the 200 pg/mL std.	300µl of the Diluent Buffer
32 pg/mL	200µl of the 80 pg/mL std.	300µl of the Diluent Buffer
12.8 pg/mL	200µl of the 32 pg/mL std.	300µl of the Diluent Buffer
5.12 pg/mL	200µl of the 12.8 pg/mL std.	300µl of the Diluent Buffer
2.0 pg/mL	200µl of the 5.12 pg/mL std.	300µl of the Diluent Buffer
0 pg/mL	200µl mL of the 2.0 pg/mL std.	An empty tube

5. The wash concentrate (20x) was examined for visible crystals, warmed to room temperature and mixed gently until dissolved. 20ml of wash buffer concentrate was diluted into deionised or distilled water to yield 400ml of 1x wash buffer.

6. The detection antibody was briefly spun before use. 100µl of assay diluents was added to the vial to prepare a detection antibody concentrate. This mixture was pipetted up and down to mix gently. The detection antibody concentrate was diluted 80 fold with 1x assay diluent.

7. The HRP-streptavidin concentrate vial was briefly spun and pipetted up and down to mix gently before use. HRP-streptavidin concentrate was diluted 400 fold with 1x assay diluent ie. 30µl of HRP-streptavidin concentrate was added into a tube with 12ml 1x assay diluent to prepare a final 400 fold diluted HRP- streptavidin solution.

MCP-1 ELISA Assay method

1. Bring all reagents and samples to room temperature (18-25°C) before use.

2. Add 100µl of each standard and samples into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or overnight at 4°C with gently shaking.

3. Discard the solution and wash 4 times with 1x wash solution. Wash by filling each well with wash buffer (300µl) using a multichannel pipette. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100µl of 1x prepared biotinylated antibody to each well. Incubate for 1 hour at room temperature with gently shaking.

5. Discard the solution. Repeat the wash step as in step 3.

6. Add 100µl of prepared streptavidin solution to each well. Incubate at 45 minutes at room temperature with gentle shaking.

7. Discard the solution. Repeat the wash step as in step.

8. Add 100µl of TMB one-step substrate reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

9. Add 50µl of stop solution to each well. Read at 450nm immediately.

APPENDIX B

Detailed protocol for reagent preparation

Virkon- purchased as a powder from Fisher Scientific (UK). Store packs dry at room temperature.

For protocol: make a 2x concentrate solution. This is a 2% stock solution in tap water for disinfection (ie 2g per 100ml). This is equivalent to 1 spoonful of Virkon in 500ml.

PBS

1 tablet added to 500ml distilled water and autoclaved

Media

Dulbecco's modified eagle medium (DMEM GlutaMAX, Gibco, Invitrogen) supplemented with 10% Foetal bovine serum (FBS, Gibco, Invitrogen), 1% Pen-Strep (5000 units/ml penicillin and 5000µl/ml streptomycin, Gibco, Invitrogen)

Glycated albumin

Main solution (10mg/ml): 25mg + 2.5ml PBS (Sigma Aldrich, UK)

Stock solution (2mg/ml): 300µl main solution + 1200µl PBS

Albumin

Main solution (100mg/ml): 5g albumin + 50ml PBS (Sigma Aldrich, UK)

Stock solution (2mg/ml): 30µl main solution + 1470µl PBS

Recombinant human interleukin-1β

Delivered as 10µg (10000ng) (Merck Millipore, UK)

Centrifuged briefly

Reconstituted with dH_2O to 100µl: equivalent to 100ng/µl

Aliquoted into 2µl (200ng) samples and stored at -20°C

For use: sample aliquots defrosted and diluted x10

2µl sample + 18µl PBS equivalent to 20µl (200ng)

2µl diluted sample equivalent to 20ng

Recombinant human TNF-α

Delivered as 50µg (50000ng) (Merck Millipore, UK)

Centrifuged briefly

Reconstituted with dH_2O to 100µl: equivalent to 500ng/µl

Aliquots of 4µl (2000ng) stored at -20°C

For use:

Samples defrosted and diluted x100

4µl sample + 396µl PBS equivalent to 20ng

Inhibitor preparations

Ly294002- specific inhibitor of P13k pathway

1mg MW 343.80 (Sigma Aldrich, UK)

DMSO soluble >5mg/ml: require 50µM

Add 1mg to 29ml DMSO equivalent to stock solution of 100µM/ml

For 50µM use 500µl stock solution

Cells pretreated for 1 hour then SFM changed and 500mg GHSA added for 24 hours

SB202190- p38 inhibitor

5mg MW 331.34 (Sigma Aldrich, UK)

DMSO soluble 30mg/ml: require 30µM

Add 5mg to 50ml DMSO equivalent to stock solution of 300µM/ml

For 30µM use 100µl stock solution

Cells pretreated for 1 hour then SFM changed and 500mg GHSA added for 24 hours

JSH-23- p65 subunit of NF-κB inhibitor

5mg MW 240.34 (Sigma Aldrich, UK)

DMSO soluble >10mg/ml: require 50µM

Add 5mg to 41.5ml DMSO equivalent to stock solution of 500µM/ml

For 50µM use 100µl stock solution

Cells pretreated for 1 hour then SFM changed and 500mg GHSA added for 24 hours

Appendix C: Statistical results

Figure 12: Effects of minocycline and hypoxia on ARPE-19 cell viability

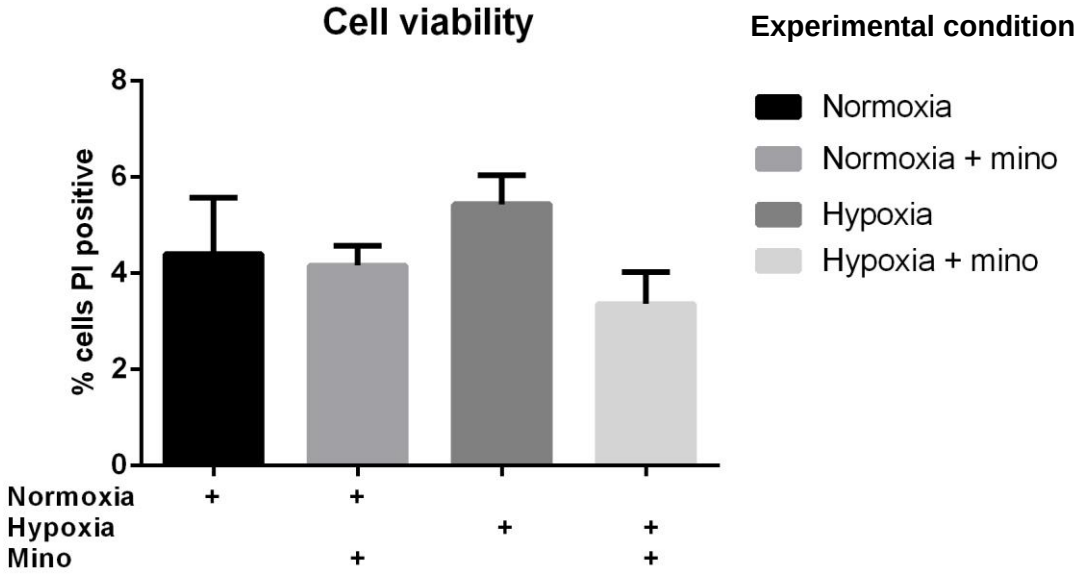

Table 9: Statistical results relating to Figure 12

Row	Experimental condition	Mean %	SD	N
A	Normoxia	4.4	1.18	3
B	Normoxia + Mino	4.2	0.40	3
C	Hypoxia	5.4	0.61	3
D	Hypoxia + Mino	3.4	0.67	3

Kruskal-Wallis test

Kruskal-Wallis statistic : 6.371
P value: 0.0787
Do the medians vary significantly (P < 0.05) : No
P value summary: Not significant

Table 10: Dunn's multiple comparison test of data relating to Figure 12

	Mean rank difference	Significant	Summary
Row A and B	0.6667	No	NS
Row A and C	-3.667	No	NS
Row A and D	3.667	No	NS
Row B and C	-4.333	No	NS
Row B and D	3.000	No	NS
Row C and D	7.333	No	NS

Figure 13: Effects of minocycline and hypoxia on ARPE-19 cell apoptosis

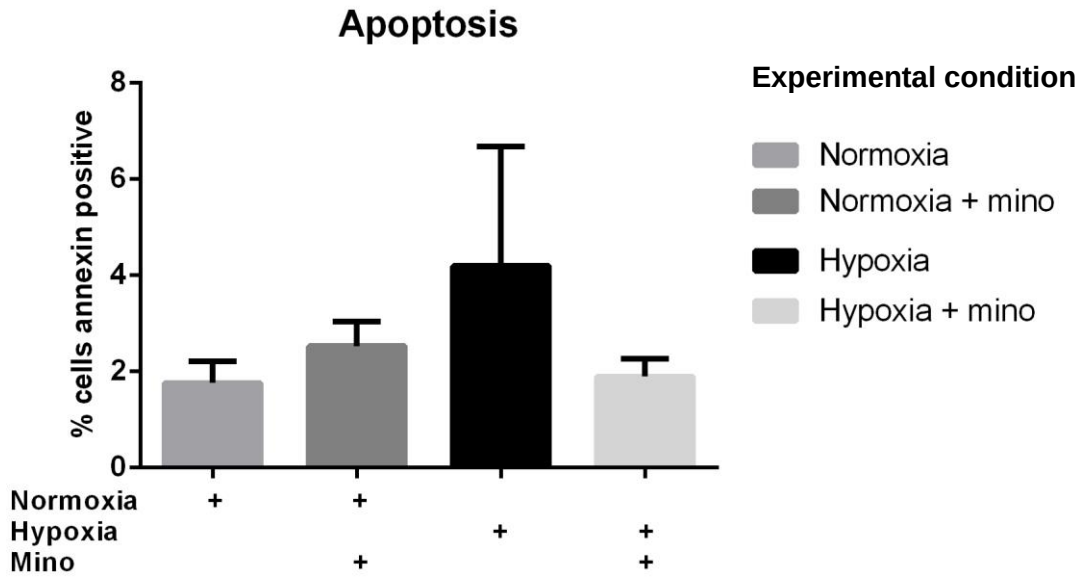

Table 11: Statistical results relating to Figure 13

Row	Experimental condition	Mean %	SD	N
A	Normoxia	4.2	2.48	3
B	Normoxia + Mino	1.8	0.45	3
C	Hypoxia	2.5	0.51	3
D	Hypoxia + Mino	1.9	0.36	3

Kruskal-Wallis test
Kruskal-Wallis statistic: 6.830
P value: 0.0521
Do the medians vary significantly (P < 0.05): No
P value summary: Not significant

Table 12: Dunn's multiple comparison test of data relating to Figure 13

	Mean rank difference	Significant	Summary
Row A and B	6.667	No	NS
Row A and C	2.167	No	NS
Row A and D	5.833	No	NS
Row B and C	-4.500	No	NS
Row B and D	-0.8333	No	NS
Row C and D	3.667	No	NS

Figure 14: Effects of glycated albumin on ARPE-19 cell viability after exposure to minocycline and hypoxia

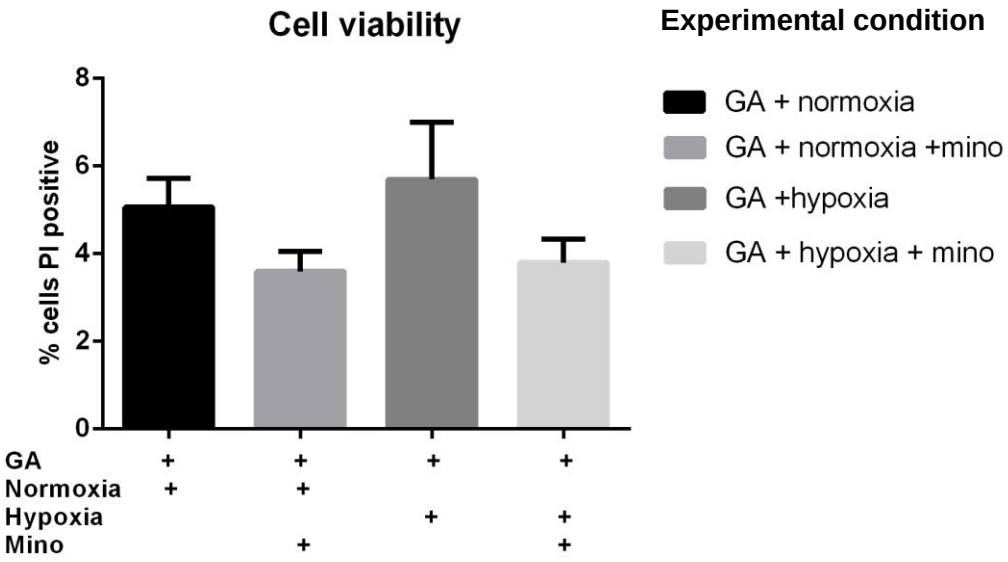

Table 13: Statistical results relating to Figure 14

Row	Experimental condition	Mean %	SD	N
A	GA + Normoxia	5.1	0.65	3
B	GA +Normoxia + Mino	3.9	0.36	3
C	GA + Hypoxia	5.7	1.3	3
D	GA + Hypoxia + Mino	3.8	0.53	3

Kruskal-Wallis test
Kruskal-Wallis statistic: 6.714
P value: 0.0601
Do the medians vary significantly (P < 0.05): No
P value summary: Not significant

Table 14: Dunn's multiple comparison test of data relating to Figure 14

	Mean rank difference	Significant	Summary
Row A and B	4.667	No	NS
Row A and C	-0.6667	No	NS
Row A and D	5.333	No	NS
Row B and C	-5.333	No	NS
Row B and D	0.6667	No	NS
Row C and D	6.000	No	NS

Figure 15: Effects of glycated albumin on ARPE-19 cell apoptosis after exposure to minocycline and hypoxia

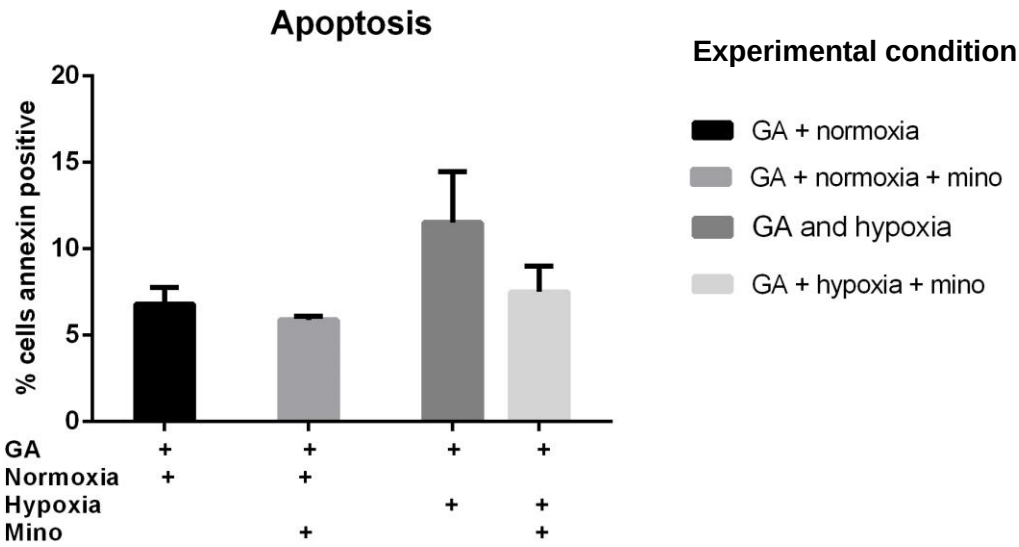

Table 15: Statistical results relating to Figure 15

Row	Experimental condition	Mean %	SD	N
A	GA + Normoxia	6.8	0.95	3
B	GA +Normoxia + Mino	5.9	0.20	3
C	GA + Hypoxia	11.5	2.93	3
D	GA + Hypoxia + Mino	7.5	1.49	3

Kruskal-Wallis test
Kruskal-Wallis statistic: 6.832
P value: 0.0506
Do the medians vary significantly ($P < 0.05$): No
P value summary: Not significant

Table 16: Dunn's multiple comparison test of data relating to figure 15

	Mean rank difference	Significant	Summary
Row A and B	2.333	No	NS
Row A and C	-5.167	No	NS
Row A and D	-1.167	No	NS
Row B and C	-7.500	No	NS
Row B and D	-3.500	No	NS
Row C and D	4.000	No	NS

Figure 17: Effects of hypoxia and minocycline on glycated albumin induced IL-8 production

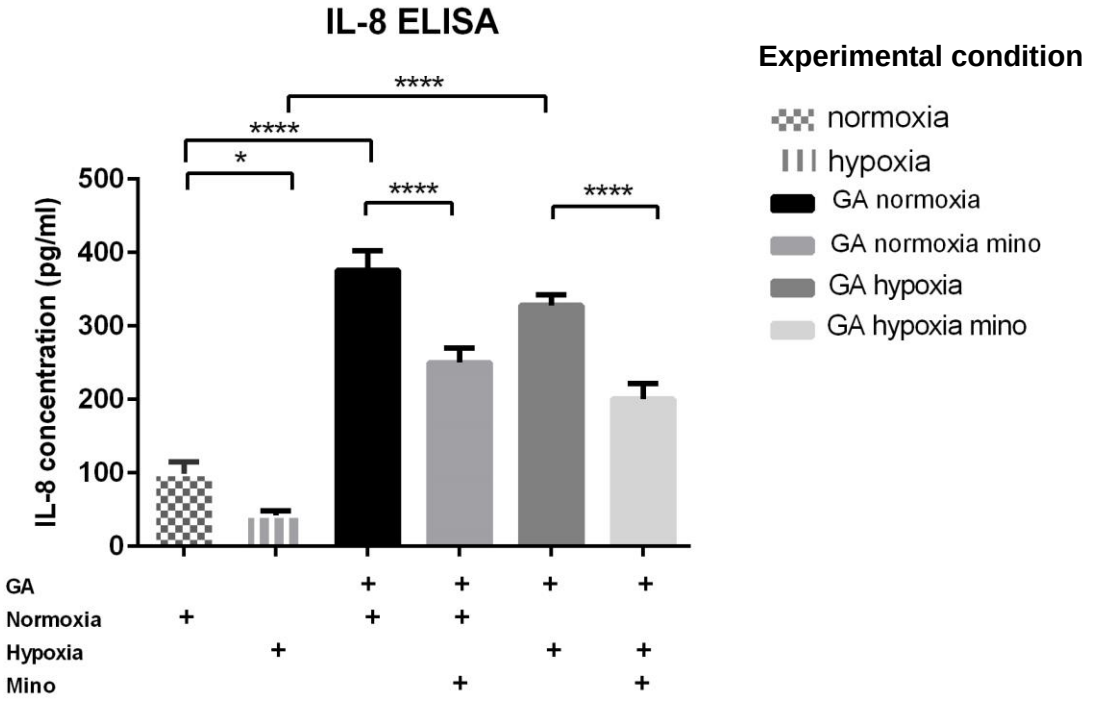

Table 17: Statistical results relating to Figure 17

IL-8 ELISA	Experimental condition	Mean Conc.	SD	N
A	normoxia	98.78	15.96	3
B	hypoxia	42.41	6.29	3
C	GA normoxia	375.89	26.76	3
D	GA normoxia + mino	250.15	19.70	3
E	GA hypoxia	328.05	14.04	3
F	GA hypoxia + mino	200.57	20.75	3

ANOVA summary

F value: 148.1

P value: < 0.0001

Are differences between means statistically different? (p<0.05): Yes

R square: 0.9841

Table 18: Tukey's multiple comparison test of data relating to Figure 17

Test details	Mean difference	95% CI of difference	Significant?	Summary
Row A and B	56.37	5.966 to 106.8	Yes	*
Row A and C	-277.1	-327.5 to -226.7	Yes	****
Row A and D	-151.4	-201.8 to -101.0	Yes	****
Row A and E	-229.3	-279.7 to -178.9	Yes	****
Row A and F	-101.8	-152.2 to -51.39	Yes	***
Row B and C	-333.5	-383.9 to -283.1	Yes	****
Row B and D	-207.7	-258.1 to -157.3	Yes	****
Row B and E	-285.6	-336.0 to -235.2	Yes	****
Row B and F	-158.2	-208.6 to -107.8	Yes	****
Row C and D	125.7	75.34 to 176.1	Yes	****
Row C and E	47.84	-2.564 to 98.24	No	NS
Row C and F	175.3	124.9 to 225.7	Yes	****
Row D and E	-77.90	-128.3 to -27.50	Yes	**
Row D and F	49.58	-0.8244 to 99.98	No	NS
Row E and F	127.5	77.08 to 177.9	Yes	****

Figure 18: Effects of Minocycline on IL-1β stimulated IL-8 production

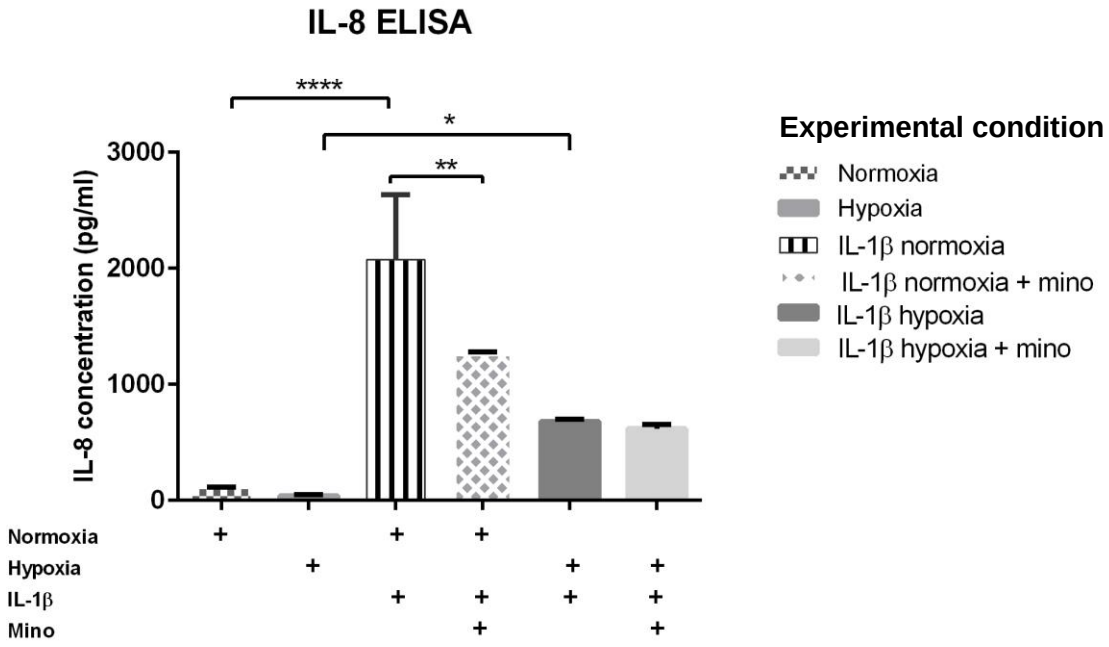

Table 19: Statistical results relating to Figure 18

IL-8 ELISA	Experimental condition	Mean Conc.	SD	N
A	Normoxia	98.78	15.96	3
B	Hypoxia	42.41	6.29	3
C	Normoxia + IL-1β	2075.7	558.5	3
D	Normoxia + IL-1β + Mino	1267.23	11.49	3
E	Hypoxia + IL-1β	681.94	15.74	3
F	Hypoxia + IL-1β + Mino	617.81	36.62	3

ANOVA summary

F value: 33.92

P value: <0.0001

Are differences between means statistically significant? (p<0.05): Yes

R square: 0.9339

Table 20: Tukey's multiple comparisons test of data relating to Figure 18

Test details	Mean difference	95% CI of difference	Significant?	Summary
Row A and B	56.37	-571.0 to 683.8	No	NS
Row A and C	-1977	-2604 to -1350	Yes	****
Row A and D	-1168	-1796 to -541.1	Yes	***
Row A and E	-583.2	-1211 to 44.23	No	NS

Row A and F	-519.0	-1146 to 108.4	No	NS
Row B and C	-2033	-2661 to -1406	Yes	****
Row B and D	-1225	-1852 to -597.4	Yes	***
Row B and E	-639.5	-1267 to -12.14	Yes	*
Row B and F	-575.4	-1203 to 51.99	No	NS
Row C and D	808.5	181.1 to 1436	Yes	**
Row C and E	1394	766.4 to 2021	Yes	****
Row C and F	1458	830.5 to 2085	Yes	****
Row D and E	585.3	-42.10 to 1213	No	NS
Row D and F	649.4	22.03 to 1277	Yes	*
Row E and F	64.13	-563.3 to 691.5	No	NS

Figure 19: Effects of individual biochemical inhibitors on glycated albumin IL-8 production from ARPE-19 cells

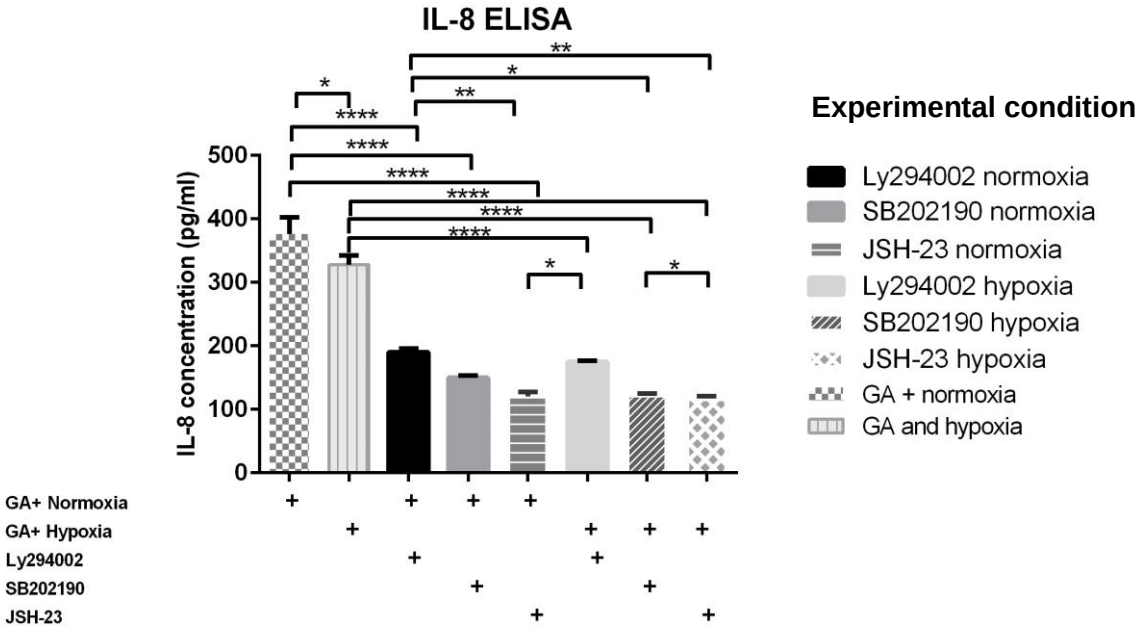

Table 21: Statistical results relating to Figure 19

IL-8 ELISA	Experimental condition	Mean Conc.	SD	N
A	GA and normoxia	375.89	26.77	3
B	GA and hypoxia	328.05	14.04	3
C	Ly294002 + GA normoxia	190.09	5.97	2
D	SB202190 + GA normoxia	149.86	3.18	2
E	JSH-23 + GA normoxia	120.68	6.99	2
F	Ly294002 + GA	174.78	2.56	2

	hypoxia			
G	SB202190 + GA hypoxia	122.58	2.59	2
H	JSH-23 + GA hypoxia	116.79	4.15	2

ANOVA summary

F value: 134.1

P value: < 0.0001

Are differences among means statistically significant? (p<0.05): Yes

R square: 0.9895

Table 22: Tukey's multiple comparison tests of data relating to Figure 19

Test details	Mean difference	95% CI of difference	Significant?	Summary
Row A and B	47.84	5.068 to 90.61	Yes	*
Row A and C	185.8	138.0 to 233.6	Yes	****
Row A and D	226.0	178.2 to 273.9	Yes	****
Row A and E	255.2	207.4 to 303.0	Yes	****
Row A and F	201.1	153.3 to 248.9	Yes	****
Row A and G	253.3	205.5 to 301.1	Yes	****
Row A and H	259.1	211.3 to 306.9	Yes	****
Row B and C	138.0	90.13 to 185.8	Yes	****
Row B and D	178.2	130.4 to 226.0	Yes	****
Row B and E	207.4	159.5 to 255.2	Yes	****
Row B and F	153.3	105.4 to 201.1	Yes	****
Row B and G	205.5	157.6 to 253.3	Yes	****
Row B and H	211.3	163.4 to 259.1	Yes	****
Row C and D	40.24	-12.15 to 92.62	No	NS
Row C and E	69.42	17.03 to 121.8	Yes	**
Row C and F	15.31	-37.07 to 67.69	No	NS
Row C and G	67.52	15.13 to 119.9	Yes	*
Row C and H	73.30	20.92 to 125.7	Yes	**
Row D and E	29.18	-23.20 to 81.56	No	NS
Row D and F	-24.93	-77.31 to 27.46	No	NS
Row D and G	27.28	-25.10 to 79.66	No	NS
Row D and H	33.07	-19.32 to 85.45	No	NS
Row E and F	-54.11	-106.5 to -1.720	Yes	*
Row E and G	-1.900	-54.28 to 50.48	No	NS
Row E and H	3.885	-48.50 to 56.27	No	NS
Row F and G	52.21	-0.1799 to 104.6	No	NS
Row F and H	57.99	5.605 to 110.4	Yes	*
Row G and H	5.785	-46.60 to 58.17	No	NS

Figure 20: Effects of combination of biochemical inhibitors on glycated albumin induced IL-8 production

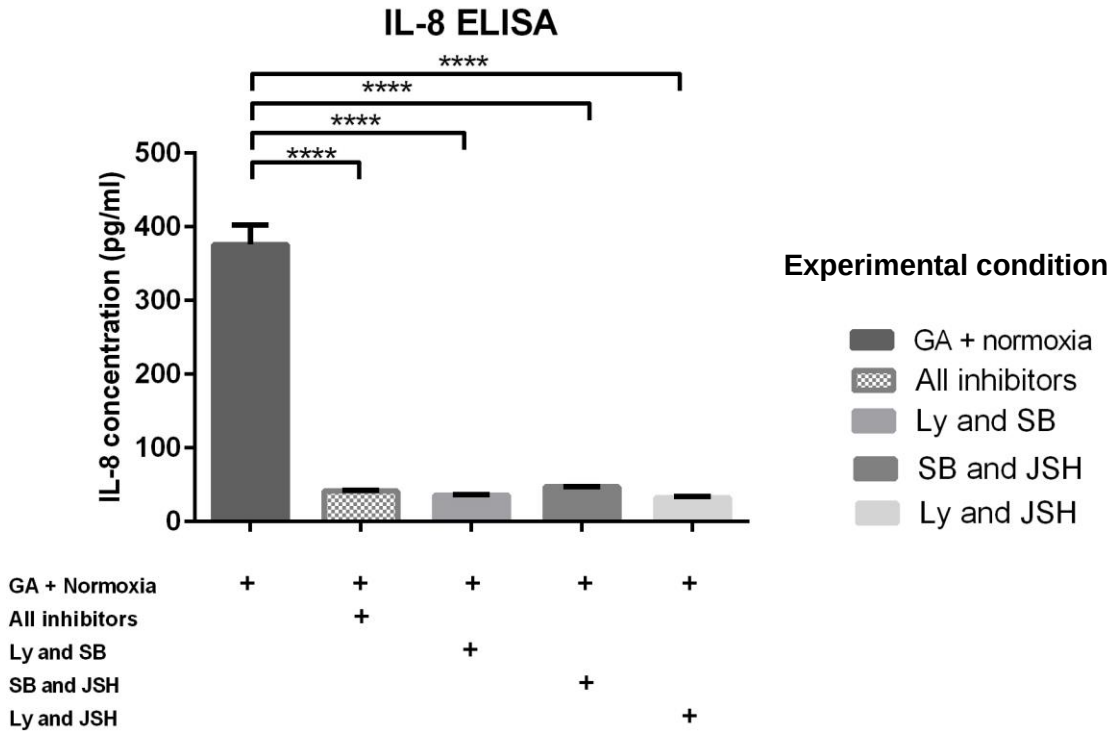

Table 23: Statistical results relating to Figure 20

IL-8 ELISA	Experimental condition	Mean Conc.	SD	N
A	GA + normoxia	375.89	26.77	3
B	All inhibitors GA + normoxia	41.65	1.0112	2
C	Ly294002 + SB202190 GA + normoxia	36.215	0.347	2
D	SB202190 + JSH-23 GA + normoxia	47.26	0.23	2
E	Ly294002 + JSH-23 GA + normoxia	32.795	1.549	2

ANOVA summary

F value: 258.1

P value: < 0.0001

Are differences among means statistically significant? (p<0.05): Yes

R square: 0.9942

Table 24: Tukey's multiple comparisons test of data relating to Figure 20

Test details	Mean Difference	95% CI of difference	Significant?	Summary
Row A and B	334.2	281.3 to 387.2	Yes	****
Row A and C	339.7	286.7 to 392.7	Yes	****
Row A and D	328.6	275.6 to 381.6	Yes	****
Row A and E	343.1	290.1 to 396.1	Yes	****
Row B and C	5.435	-52.61 to 63.48	No	NS
Row B and D	-5.610	-63.65 to 52.43	No	NS
Row B and E	8.855	-49.19 to 66.90	No	NS
Row C and D	-11.05	-69.09 to 47.00	No	NS
Row C and E	3.420	-54.62 to 61.46	No	NS
Row D and E	14.47	-43.58 to 72.51	No	NS

Figure 22: Effects of hypoxia and minocycline on glycated albumin induced MCP-1 production

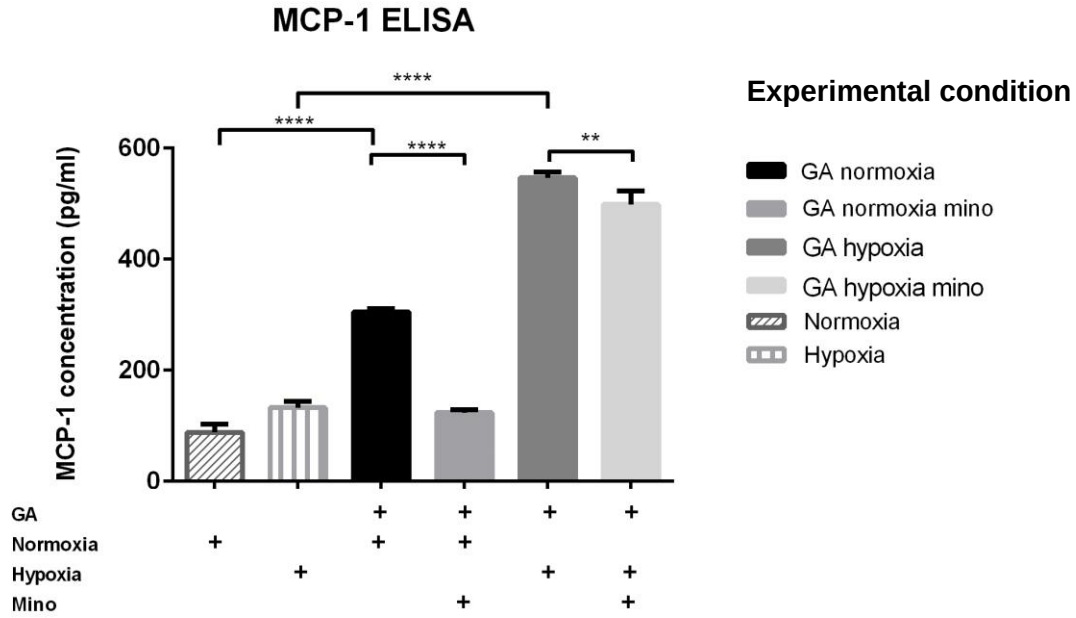

Table 25: Statistical results relating to Figure 22

MCP-1 ELISA	Experimental condition	Mean Conc.	SD	N
A	Normoxia	88.38	14.62	3
B	Hypoxia	132.8	10.7	3
C	GA normoxia	305.45	5.88	3
D	GA normoxia + mino	123.67	5.27	3
E	GA hypoxia	546.66	10.69	3
F	GA hypoxia + mino	498.58	24.06	3

ANOVA summary

F value: 671.7

P value: <0.0001

Are differences between means statistically significant? (p<0.05): Yes

R square: 0.9964

Table 26: Tukey's multiple comparisons test of data relating to Figure 22

Test details	Mean difference	95% CI of difference	Significant?	Summary
Row A and B	-44.42	-81.29 to -7.552	Yes	*
Row A and C	-217.1	-253.9 to -180.2	Yes	****

Row A and D	-35.29	-72.16 to 1.578	No	NS
Row A and E	-458.3	-495.1 to -421.4	Yes	****
Row A and F	-410.2	-447.1 to -373.3	Yes	****
Row B and C	-172.7	-209.5 to -135.8	Yes	****
Row B and D	9.130	-27.74 to 46.00	No	NS
Row B and E	-413.9	-450.7 to -377.0	Yes	****
Row B and F	-365.8	-402.6 to -328.9	Yes	****
Row C and D	181.8	144.9 to 218.6	Yes	****
Row C and E	-241.2	-278.1 to -204.3	Yes	****
Row C and F	-193.1	-230.0 to -156.3	Yes	****
Row D and E	-423.0	-459.9 to -386.1	Yes	****
Row D and F	-374.9	-411.8 to -338.0	Yes	****
Row E and F	48.08	11.21 to 84.95	Yes	**

Figure 23: Effects of individual biochemical inhibitors on glycated albumin stimulated MCP-1 production from ARPE-19 cells

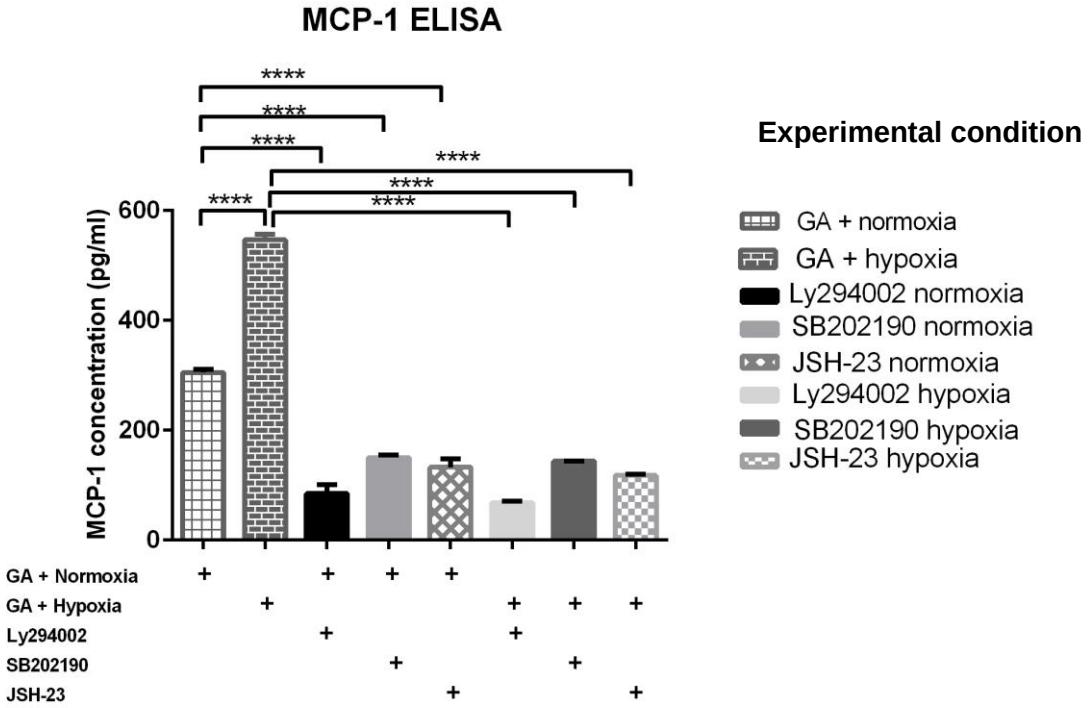

Table 27: Statistical results relating to Figure 23

MCP-1 ELISA	Experimental condition	Mean Conc.	SD	N
A	GA and normoxia	305.45	5.87	3
B	GA and hypoxia	546.66	10.69	3
C	Ly294002 + GA normoxia	84.94	16.24	2
D	SB202190 + GA normoxia	150.01	4.89	2
E	JSH-23 + GA normoxia	132.84	14.86	2
F	Ly294002 + GA hypoxia	68.07	3.19	2
G	SB202190 + GA hypoxia	143.58	0.91	2
H	JSH-23 + GA hypoxia	117.59	2.43	2

ANOVA summary

F value: 837.3

P value: <0.0001

Are differences between means statistically significant? (p<0.05): Yes

R square: 0.9

Table 28: Tukey's multiple comparison tests of data relating to Figure 23

Test details	Mean difference	95% CI of difference	Significant?	Summary
Row A and B	-241.2	-269.0 to -213.4	Yes	****
Row A and C	220.5	189.4 to 251.6	Yes	****
Row A and D	155.4	124.4 to 186.5	Yes	****
Row A and E	172.6	141.5 to 203.7	Yes	****
Row A and F	237.4	206.3 to 268.4	Yes	****
Row A and G	161.9	130.8 to 192.9	Yes	****
Row A and H	187.9	156.8 to 218.9	Yes	****
Row B and C	461.7	430.7 to 492.8	Yes	****
Row B and D	396.7	365.6 to 427.7	Yes	****
Row B and E	413.8	382.8 to 444.9	Yes	****
Row B and F	478.6	447.5 to 509.7	Yes	****
Row B and G	403.1	372.0 to 434.1	Yes	****
Row B and H	429.1	398.0 to 460.1	Yes	****
Row C and D	-65.07	-99.09 to -31.05	No	***
Row C and E	-47.90	-81.92 to -13.88	Yes	**
Row C and F	16.87	-17.15 to 50.89	No	NS
Row C and G	-58.64	-92.66 to -24.62	Yes	**
Row C and H	-32.65	-66.67 to 1.375	Yes	NS
Row D and E	17.17	-16.85 to 51.19	No	NS
Row D and F	81.94	47.92 to 116.0	No	****
Row D and G	6.430	-27.59 to 40.45	No	NS
Row D and H	32.42	-1.605 to 66.44	No	NS
Row E and F	64.77	30.75 to 98.79	Yes	***
Row E and G	-10.74	-44.76 to 23.28	No	NS
Row E and H	15.25	-18.77 to 49.27	No	NS
Row F and G	-75.51	-109.5 to -41.49	No	***
Row F and H	-49.52	-83.54 to -15.50	Yes	**
Row G and H	25.99	-8.035 to 60.01	No	NS

Figure 24: Effects of combination of biochemical inhibitors on glycated albumin induced MCP-1 production

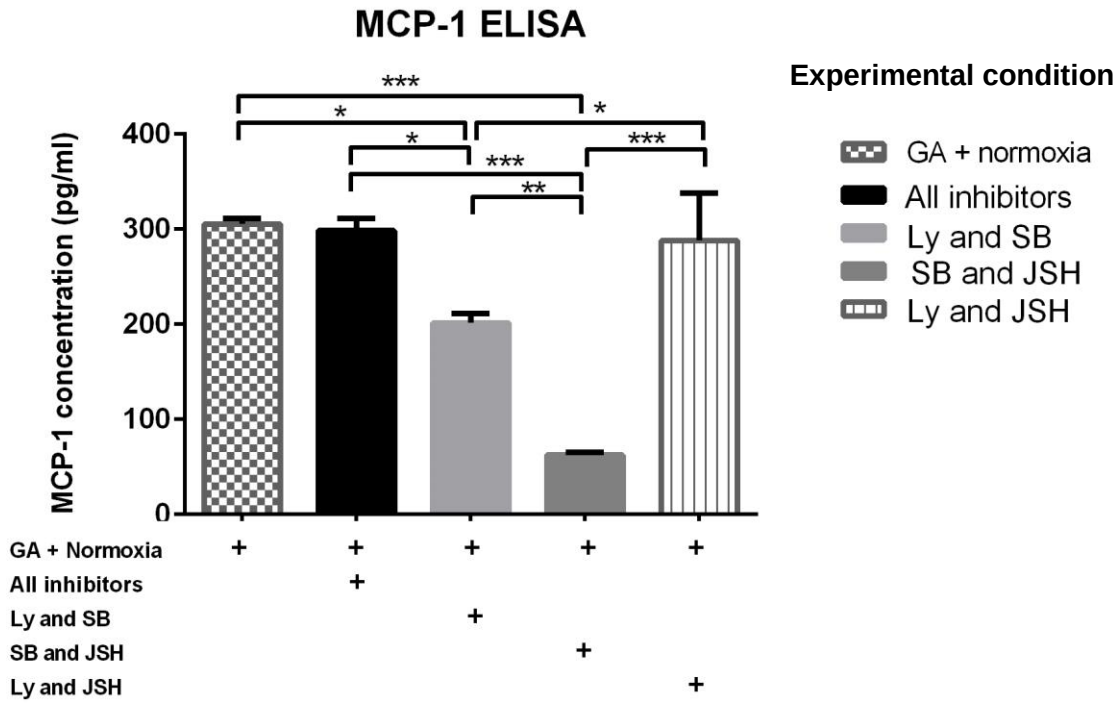

Table 29: Statistical results relating to Figure 24

MCP-1 ELISA	Experimental condition	Mean Conc.	SD	N
A	GA + normoxia	305.45	5.87	3
B	All inhibitors GA + normoxia	298.34	13.11	2
C	Ly294002 + SB202190 GA + normoxia	201.49	9.84	2
D	SB202190 + JSH-23 GA + normoxia	62.42	3.05	2
E	Ly294002 + JSH-23 GA + normoxia	288.27	49.97	2

ANOVA summary

F value: 47.63

P value: 0.0001

Are differences among means statistically significant? ($p<0.05$): Yes

R square: 0.9695

Table 30: Tukey's multiple comparison tests of data relating to Figure 24

Test details	Mean Difference	95% CI of difference	Significant?	Summary
Row A and B	7.110	-67.44 to 81.66	No	NS
Row A and C	104.0	29.41 to 178.5	Yes	*
Row A and D	243.0	168.5 to 317.6	Yes	***
Row A and E	17.18	-57.37 to 91.73	No	NS
Row B and C	96.85	15.18 to 178.5	Yes	*
Row B and D	235.9	154.3 to 317.6	Yes	***
Row B and E	10.07	-71.60 to 91.74	No	NS
Row C and D	139.1	57.40 to 220.7	Yes	**
Row C and E	-86.78	-168.4 to -5.111	Yes	*
Row D and E	-225.9	-307.5 to -144.2	Yes	***

Table 31: Effects of IL-1β induced MCP-1 production

MCP-1 ELISA	Experimental condition	Mean Conc.	SD	N
A	Normoxia	546.66	10.69	3
B	Hypoxia	498.58	24.06	3
C	IL-1β normoxia	722.56	221.16	3
D	IL-1β normoxia + mino	537.66	152.13	3
E	IL-1β hypoxia	536.06	60.17	3
F	IL-1β hypoxia + mino	345.75	45.35	3

ANOVA summary

F value: 3.322

P value: 0.0410

P value: Are differences among means statistically significant? ($p<0.05$): Yes

R square: 0.5806

Table 32: Tukey's multiple comparisons test of data relating to effects of IL-1β induced MCP-1 production

Test details	Mean difference	95% CI of difference	Significant?	Summary
Row A and B	48.08	-265.5 to 361.6	No	NS
Row A and C	-175.9	-489.5 to 137.7	No	NS
Row A and D	9.000	-304.6 to 322.6	No	NS

Row A and E	10.60	-303.0 to 324.2	No	NS
Row A and F	200.9	-112.6 to 514.5	No	NS
Row B and C	-224.0	-537.5 to 89.57	No	NS
Row B and D	-39.08	-352.6 to 274.5	No	NS
Row B and E	-37.48	-351.0 to 276.1	No	NS
Row B and F	152.8	-160.7 to 466.4	No	NS
Row C and D	184.9	-128.7 to 498.5	No	NS
Row C and E	186.5	-127.1 to 500.1	No	NS
Row C and F	376.8	63.26 to 690.4	Yes	*
Row D and E	1.600	-312.0 to 315.2	No	NS
Row D and F	191.9	-121.6 to 505.5	No	NS
Row E and F	190.3	-123.2 to 503.9	No	NS

Table 33: Data relating to Figure 11 Concentration dependent effects of Minocycline on ARPE-19 cell viability

Minocycline concentration (μM)	Mean percentage of viable cells	Standard deviation	N (number of independent experiments
0	96	4	3
5	94	5	3
10	86	6	3
15	80	8	3
20	63	10	3

Appendix D

PATIENT INFORMATION SHEET
Lead Researcher: Sobha Sivaprasad
King's protocol number: 06NB37

1. **Study title**

Combination therapy of Visudyne, Minocycline, Dexamethasone and Ranibizumab (VIMDER) for the treatment of subfoveal CNV

2. **Invitation paragraph**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you fo reading this.

3. **What is the purpose of the study?**

To find out the effectiveness of combining for drugs to treat wet AMD.
The medications that will be used are:

1) Photodynamic therapy (PDT) is the standard treatment in the NHS for your condition
2) Ranibizumab (Lucentis)- this drug blocks a molecule that is thought to be involved in the growth of new blood vessels as well as their leakage in the retina. This is not yet available on the NHS. It has been used in several research studies and found to be useful in stabilising vision.
3) Dexamethasone- a steroid that helps to settle inflammation
4) Minocycline- is commonly used as a treatment for acne. It helps to block the molecules that encourage blood vessels to grow.

4. **Why have I been chosen?**

You have been asked to take part in this study because you have wet AMD. This has caused the changes that you have noticed in your eyesight. Small abnormal blood vessels behind the retina leak fluid and blood into the central area of the retina causing it to malfunction, scar tissue also develops. If left untreated it may cause you to lose the central vision permanently.

5. **Do I have to take part?**

No- it is up to you to decide whether or not you take part. If you do decide to take part you will be given this information sheet to read and keep. You will be assessed to see if you are suitable and be asked to sign a consent form. Allergy to any of the treatments will obviously mean you cannot take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. Your future treatment and care will not be affected.

6. **What will happen to me if I take part?**

The study will last for 1 year. You will be asked to come to the clinic monthly for the first 12 months. At each visit you vision will be tested, your eye examined and a scan performed of the back of the eye. Fluorescein angiogram will be performed at the first visit only.

Treatment

First treatment visit

Photodynamic therapy. An intravenous injection is given over 10 minutes and then a laser is applied with a contact lens over 2 minutes.

Dexamethasone/ Ranibizumab injection. These two drugs are applied by one injection into the eye. This treatment takes around 10 minutes and for this you will lie on a bed. You will need to apply antibiotic drops for four days after this.

You will be given Minocycline tablets to be taken every day for three months.

Follow up treatment visits

You may not require treatment after the first visit, this will depend on the results of the examination and scan. If further treatment is needed it will be an injection of ranibizumab only.

7. What are the side effects of taking part?

Study medication

The injection of dexamethasone and Ranibizumab can cause transient discomfort during the procedure. There may be some redness, this usually settles in a few days. In a few cases there can be a mild inflammation of the eye. The injection has a low rate (less than 1%) of serious complication such as infection, haemorrhage or retinal detachment. A mild increase in the pressure inside the eye may occur. All these can be treated if develops.

Photodynamic therapy

A small number of patients (less than 5%) have dull back pain during the infusion, this passes off after a couple of minutes. A few patients (less than 2%) have a temporary visual loss during the first week after PDT. Most patients recover within four weeks without treatment.

Problems or side effects such as reactions to the medications may arise. Should any problems occur, you will be given appropriate care for your condition, and you may be withdrawn from the study.

Other possible risks

Fluorescein angiogram is a safe test routinely used in investigating the eye. Before the pictures of the eye are taken, you will receive an injection into an arm vein of fluorescein dye. There may be minor discomfort from the needle in your arm. Common side effects of the dye are nausea and vomiting.

There is a low risk of serious allergic reaction. The dye stains your skin and urine for approximately 36 hours.
Ranibizumab is provided for 12 dosages only, and this might last between 1 to 2 years. If after 12 dosages are finished, the study will not provide any further treatment. The management will revert back to standard NHS care.

8. What are the possible benefits of taking part?

We hope that the treatment will help you. However, this cannot be guaranteed. The information we get from this study is intended to help us design and perform further similar large studies in the future with the intention of treating wet AMD better in the long term.

9. What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research the doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

10. What happens when the research study stops?

After the trial has ended, your follow up will continue in the normal clinical setting with regular follow up in your clinic.

11. What if something goes wrong?

Overall the treatment procedures have been shown to be extremely safe and we do not expect any health problems to arise as a result of taking part in this trial. However, if you have any complaints or concerns regarding the treatment you have received, this can be pursued through the normal NHS mechanisms. No compensation is available for non negligent harm.

12. Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital will have your name and address removed so that you cannot be recognised from it. With your consent, we would like to inform your GP of your participation in the trial.

13. Who is organising and funding the research?

The research is being supported partly by Novartis (the drug company which made the drug used in PDT and Lucentis). The money is used to fund tests needed for the research.

14. Who has reviewed the study?

The study has been approved by the King's College Hospital Research Ethics Committee.

15. Contact for Further Information

Please ask any questions you have about the study before deciding to take part. If you wish to contact someone once you have left the hospital about the study please contact the doctor below who will be able to answer any questions you may have.

Consent Form

Combination therapy of Visudyne, Minocycline, Dexamethasone and Ranibizumab (VIMDER) in for the treatment of subfoveal CNV.

Lead Researcher: Sobha Sivaprasad

King's protocol number: **06NB37**

Patient Trial Number:

1. I confirm that I have read and understand the information sheet dated 10th July 2008 (Version 4.1) for the above study and have had the opportunity to ask questions. I agree to take part in this study. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3.I understand that sections of any of my medical notes may be looked at by responsible individuals authorised by the lead researcher or from regulatory authorities where it is releveant to my taking part in research. I give permission for these individuals to have access to my records. ☐

Print Name..... Signature.....

Date...../...../...../

Explanation given by:

.....

NB: Three copies should be made for (1) patient, (2) researcher, (3) hospital notes.

Consent form: Date: 10th July 2008

Appendix E- Ethical Approval

NHS
National Research Ethics Service

King's College Hospital Research Ethics Committee
Camberwell Building
King's College Hospital
94 Denmark Hill
London
SE5 9RS

Telephone: 0203 299 3923
Facsimile: 0203 299 5085

28 July 2008

Mr Victor Chong
Consultant Ophthalmic Surgeon
King's College Hospital
Denmark Hill
London
SE5 9RS

Dear Mr Chong

Full title of study:	**A pilot study to examine the safety and efficacy of intravitreal ranubizumab/dexamethasone administration and oral minocycline in addition to Visudyne (verteporfin) photodynamic therapy for subfoveal choroidal neovascularization secondary to age related macular degeneration:an open label trial.**
REC reference number:	**06/Q0703/172**
Protocol number:	**Version 1 dated 22nd August 2006**
EudraCT number:	**2006-004292-35**

The REC gave a favourable ethical opinion to this study on 31 January 2007.

Further notification(s) have been received from local site assessor(s) following site-specific assessment. On behalf of the Committee, I am pleased to confirm the extension of the favourable opinion to the new site(s). I attach an updated version of the site approval form, listing all sites with a favourable ethical opinion to conduct the research.

R&D approval

The Chief Investigator or sponsor should inform the local Principal Investigator at each site of the favourable opinion by sending a copy of this letter and the attached form. The research should not commence at any NHS site until approval from the R&D office for the relevant NHS care organisation has been confirmed.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

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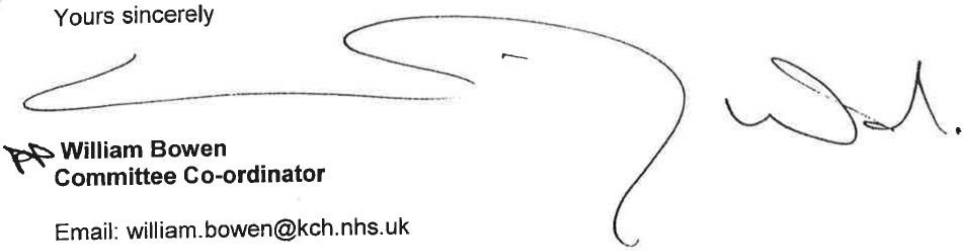
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0703/172	**Please quote this number on all correspondence**

Yours sincerely



William Bowen
Committee Co-ordinator

Email: william.bowen@kch.nhs.uk

Enclosure: *Site approval form*

Copy to: *Kings College Hospital*
Clinical Trials Unit, MHRA

King's College Hospital Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	06/Q0703/172	**Issue number:**	1	**Date of issue:**	28 July 2008
Chief Investigator:	Mr Victor Chong				
Full title of study:	A pilot study to examine the safety and efficacy of intravitreal ranubizumab/dexamethasone administration and oral minocycline in addition to Visudyne (verteporfin) photodynamic therapy for subfoveal choroidal neovascularization secondary to age related macular degeneration:an open label trial.				

This study was given a favourable ethical opinion by King's College Hospital Research Ethics Committee on 31 January 2007. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* [1]
Mr Chang		Kings College Hospital NHS Trust	King's College Hospital Research Ethics Committee	29/01/2007	
Miss Sobha Sivaprasad	Consultant Ophthalmologist	King's College Hospital NHS Foundation Trust	King's College Hospital Research Ethics Committee	28/07/2008	

Approved by the Chair on behalf of the REC:

.....(Signature of Co-ordinator)

CHRISTOPHER KIRK (Name)

[1] *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

Appendix F: Outcome of Serious adverse event

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NOVARTIS

Ms Sobha Sivaprasad
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26th August 2008

Dear Miss Sivaprasad

Investigator Notification for - Extradural haematoma "PHHO2007GB21328"

On behalf of the Novartis Integrated Medical Safety Pharmacovigilance Operations (IMS PVO) department, I enclose an Investigator Notification of a Suspected Unexpected Serious Adverse Reaction (SUSAR) dated 22nd August 2008 and entitled "Investigator Notification for ranibizumab: Extradural haematoma - PHHO2008GB21328" which is possibly related to RFB002.

For your information, this notification is being circulated to all UK and Republic of Ireland investigators participating in the RFB002 Clinical Development Programme and should be considered as supplemental data to that contained within the Investigator Brochure.

As you are the Principal Investigator in an investigator initited trial it is your responsibility to inform all your particpating centres of this investigator notification. It is also your responsibility to inform the Research Ethics Committee if necessary according to the new procedures detailed below:

NRES's Standard Operating Procedures now state that there is no requirement to notify ethics committees of SUSARs unless they occur in the Member State (ie. the UK). You do however need to provide 6 monthly and Annual Safety Reports to them listing all SUSARS that have occurred.

One copy of the Investigator Notification is enclosed. Please forward a copy to your NHS Trust or equivalent and the appropriate Ethics Committee (if required).

If you have any queries, please contact Richard Leaback on 01276 698226.

Yours sincerely

LSilverstein

Lara Silverstein
Clinical Project Manager

Please file this in relevant section of your Investigator Folder.

cc Richard Leaback

To: All Investigators in Novartis verteporfin studies and Novartis ranibizumab studies*

DATE: 22 AUG 2008

Re: Investigator Notification for verteporfin and ranibizumab studies:extradural haematoma,"

Novartis report number: PHH02007GB21328

Dear All,

In accordance with the Good Clinical Practice and specific national regulatory requirements we wish to inform you of a serious, unexpected, adverse event report of "extradural haematoma," in a patient being treated with verteporfin and ranibizumab/dexamethasone and oral minocycline during the course of a clinical study.

We would like to inform on this case which concerns an 89 year old female patient who was enrolled in study CBPD952AGB01, a open label pilot study to examine the safety and efficacy of intravitreal ranibizumab/dexamethasone administration and oral minocycline, in addition to Visudyne (verteporfin) photodynamic therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: The patient's medical history includes breast cancer, left mastectomy, transient ischemic attacks, atrial fibrillation and allergies to penicillin. The patient received study medications on 10 SEP 2007 and on 01 NOV 2007 when the last dose was administered. The patient was on concomitant Warfarin, dose and duration was not specified. On 26 NOV 2007, the patient experienced the event following a collapse and was hospitalized. An MRI of the spine revealed acute extradural bleed, with prolonged INR values which was corrected with fresh frozen plasma and vitamin K supplements. The patient died on 08 DEC 2007. Cause of death was reported to be extradural hematoma. The investigator initially submitted a report that the event was unlikely related to study drugs. Follow–up information received from the investigator indicates that the extradural hematoma to be possibly related to study drug ranibizumab. As per the Investigator Brochure and Core Data Sheet, epidural hematoma is an unlisted event and thus considered unexpected for ranibizumab and Visudyne (verteporfin) photodynamic therapy.

For the current case, details of the adverse events are provided on the attached CIOMS form, which contains the available information as reported to Novartis.

A search in Novartis database neither Visudyne nor Lucentis cases of extradural haematoma have been reported.

We will keep you informed if further medically significant information becomes available that is relevant to this event or if any additional action is necessary. We ask that you please inform your Institutional Review Board or Ethics Review Board of this event, if you have such an obligation. For clinical trials in the U.S. only, if you are utilizing the services of a central Institutional Review Board (IRB) that has been contracted through Novartis, Novartis will submit the Investigator Notification on your behalf to the central IRB.

Sincerely,

Tauqeer Karim, MD, MS, FACP, FASN

Integrated Medical Safety
Novartis Pharmaceuticals Corporation

Appendix G

Copies of relevant publications

Original Paper

Ophthalmologica

Ophthalmologica 2011;225:200–206
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Published online: February 3, 2011

A Pilot Study on the Combination Treatment of Reduced-Fluence Photodynamic Therapy, Intravitreal Ranibizumab, Intravitreal Dexamethasone and Oral Minocycline for Neovascular Age-Related Macular Degeneration

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Key Words
Verteporfin · Ranibizumab · Dexamethasone · Minocycline · Macular degeneration · Choroidal neovascularisation

Abstract
Aim: To assess the safety and efficacy of the combined treatment of reduced-fluence verteporfin photodynamic therapy (PDT), intravitreal ranibizumab, intravitreal dexamethasone and oral minocycline for choroidal neovascularisation (CNV) secondary to age-related macular degeneration (AMD). ***Methods:*** Nineteen patients with subfoveal CNV secondary to AMD were recruited into the trial. All study eyes (n = 19) received a single cycle of reduced-fluence (25 mJ/cm^2) PDT with verteporfin followed by an intravitreal injection of ranibizumab 0.3 mg/0.05 ml and dexamethasone 200 μg at baseline. Oral minocycline 100 mg daily was started the following day and continued for 3 months. Patients were followed up monthly for 12 months. Repeat intravitreal ranibizumab was given if best-corrected visual acuity (BCVA) deteriorated by >5 letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) chart or central retinal thickness (CRT) on ocular coherence tomography increased >100 μm. ***Results:*** Eighteen patients completed the 12-month study. Stable vision (loss of ≤15 ETDRS letters) was maintained in 89% eyes (16/18). The mean change in BCVA was −5.0 ± 10.5 ETDRS letters. The mean number of ranibizumab injections was 3.4 (range 2–6). The mean reduction in the CRT was 66.3 μm (±75). ***Conclusion:*** This open-label clinical trial has demonstrated the safety in terms of adverse effects and maintenance of stable vision of combination treatment with verteporfin, ranibizumab, dexamethasone and minocycline in exudative AMD. However, the outcomes with reduced-fluence PDT combination therapy does not differ significantly with outcomes of clinical trials on combination treatment with standard dose PDT and intravitreal ranibizumab in neovascular AMD.

Introduction

Age-related macular degeneration (AMD) is the commonest cause of blindness in the Western world. The exudative type is characterised by the presence of choroidal neovascularisation (CNV) [1]. The pathogenesis of CNV

Six-month data were presented at the ARVO 2008 and RCOphth Congress 2008.

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is complex and involves several intricately linked pathways including oxidative stress, inflammation, immune mechanisms and angiogenesis [2, 3]. Inhibition of vascular endothelial growth factor (VEGF) is now in widespread use for treating exudative AMD. An increasing collection of evidence suggests that immunological events play a key role in the pathogenesis of AMD. Uveitis manifests with infiltrates of inflammatory cells, oedema and tissue damage. However, in AMD the inflammatory process is less prominent but may be the common pathway culminating in visual loss.

Photodynamic therapy (PDT) was the first treatment shown to be of clinical benefit for most patients with subfoveal neovascular AMD. Standard photodynamic therapy is a 2-step process involving an initial intravenous infusion of verteporfin followed by irradiance with a 689-nm laser for 83 s beginning 5 min after the infusion delivering a total energy of 50 J/cm^2. Verteporfin binds to low-density lipoprotein receptors in the plasma during the infusion, which are then preferentially bound by choroidal neovascular tissue which expresses low-density lipoprotein receptors. Irradiation of the neovascular lesion by the laser creates toxic oxygen species that induce thrombosis and closure of the choroidal neovascularisation.

Although the thrombosis predominantly affects the choroidal neovascularisation, there is evidence of damage both to the choriocapillaris and the retinal pigment epithelium [4]. The Verteporfin in Minimally Classic CNV Study examined the effects of both reduced fluence and standard fluence in minimally classic lesions. This study suggested that reduced fluence may be beneficial in such lesions [5]. Reduced-fluence PDT with 25 J/cm^2 reduces choroidal hypoperfusion, inflammation, vascular leakage and VEGF upregulation that is associated with standard fluence PDT [1]. Reduced-fluence PDT was used only once during this study.

Anti-inflammatory agents such as intravitreal triamcinolone have been used as an adjunct for PDT to limit further VEGF upregulation initiated by the therapy.

This combination therapy has been shown to be beneficial when compared with PDT monotherapy in terms of functional results [6, 7]. However, triamcinolone has been associated with an increased risk of cataract formation [8] and raised intra-ocular pressure [9]. It was not until recently that replacement of triamcinolone with dexamethasone was considered [10]. Triamcinolone that is injected as a suspension has prolonged effects, particularly with regard to raised intra-ocular pressure. Dexamethasone which is injected as a solution is more rapidly cleared from the vitreous; as there is no sustained release due to suspension, there is reduced risk of steroid-induced side effects.

Dexamethasone has anti-inflammatory, antifibrotic and anti-VEGF effects [11–13]. Further, its antifibrotic effects are reduced in the presence of VEGF [14] so that the combination of dexamethasone with anti-VEGF therapy may assist with dexamethasone's antifibrotic effects. Dexamethasone may reduce endothelial dysfunction and inhibit VEGF-induced vascular dysfunction [11]. At the molecular level, dexamethasone exerts its anti-inflammatory effect by interfering with the activation of pro-inflammatory genes without affecting factors that inhibit inflammation [15].

Minocycline is a semisynthetic derivative of tetracycline with a longer half-life and improved penetration through the blood-brain barrier. Apart from its antimicrobial actions it also has potent anti-inflammatory and immunomodulatory effects [16]. Minocycline possesses anti-oxidant activity and inhibits both free radical production and lipid peroxidation in a concentration-dependent manner [17]. Minocycline has been shown to protect melanocytes from apoptosis-induced oxidative stress in vitiligo, a progressive disorder manifested by the selective destruction of melanocytes in the skin [18]. Leung et al. [19] investigated whether minocycline and its structurally related analogues would protect photoreceptor cells in primary bovine culture from light and oxidative stress. Minocycline was shown to protect photoreceptors in culture but within a narrow therapeutic range of concentrations. It was postulated that minocycline may act as an adjunct for neovascular AMD in the combined treatment regimen. A reduced dose of ranibizumab (0.3 mg in 0.05 ml) was used in this study given the combined angio-occlusive effect of PDT and the effects of dexamethasone and minocycline.

Combination therapy in exudative AMD is aimed at targeting the different pathways in the disease process while reducing or counteracting the adverse effects of the individual therapeutic agents. The adverse ocular effects of PDT include choroidal hypoperfusion, inflammation, upregulation of VEGF production and scarring induced by irradiance. The adverse ocular effects of ranibizumab include the potential detrimental effects of chronic VEGF blockade on the function of photoreceptors. Adverse ocular effects of dexamethasone include raised intra-ocular pressure and cataract. Minocycline as an individual agent has no specific adverse ocular effects except the rarely reported association with idiopathic intracranial hypertension. In terms of safety, potential negative synergistic effects of photosensitivity of minocycline and PDT exist.

In addition, a negative synergic effect of inducing ischaemia in the choroidal and retinal circulation followed by atrophy exists with combined PDT, intravitreal ranibizumab and dexamethasone.

The current treatment of CNV secondary to AMD is anti-VEGF monotherapy with ranibizumab [20]. Randomised clinical trials of monotherapy with the anti-VEGF agent ranibizumab (MARINA and ANCHOR) while achieving good visual outcomes (95% losing fewer than 15 letters of logMAR vision after 24 months) required monthly intravitreal injections over a 2-year period [21, 22]. The recommended clinical guidance for administering ranibizumab is monthly intravitreal injections for 3 months with additional injections based on monthly vision and optical coherence tomography (OCT) assessment [23, 24]. Not only do these treatment protocols have major implications on the health care system, but repeated ranibizumab injections can trigger an adaptive cascade that counteracts the beneficial effects of anti-VEGF blockade [25]. We hypothesised that by combining ranibizumab with other CNV-modulating agents, the need for ranibizumab retreatment could be reduced while remaining both efficacious and safe.

Methods

This was a prospective open-label interventional clinical trial of eyes with recent-onset CNV secondary to AMD.

Eligibility Criteria

The inclusion criteria were (1) age ≥50 years, (2) clinical diagnosis of wet AMD, (3) any active subfoveal CNV (classic and occult) and (4) logMAR best-corrected visual acuity (BCVA) of 24–73 letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) chart. Activity of the CNV was determined by recent decline in vision, presence of subretinal fluid or haemorrhage and leakage on fundus fluorescein angiogram. Only one eye of each patient was included in the study. The main exclusions were (1) eyes with CNV from causes other than AMD (myopia, angioid streaks, choroidal rupture, chorioretinal scarring), (2) previous treatment for CNV (PDT, argon laser, anti-VEGF agents, steroids), (3) open-angle glaucoma or risk of developing glaucoma and (4) myopia >−8.00 dpt.

Ophthalmic Assessments

The logMAR BCVA was measured in both eyes after refraction using the modified ETDRS charts. The BCVA, slit-lamp biomicroscopic examination of the anterior segment, intra-ocular pressure measurement and dilated fundus examination of the posterior pole were performed at baseline and monthly. Baseline and monthly OCT imaging was carried out on the Stratus OCT (Carl Zeiss Meditec) using the fast macular thickness map protocol. Colour fundus photography and fluorescein angiography were performed at baseline, month 6 and month 12.

Treatment Schedule at Baseline

Reduced-fluence PDT was administered at baseline. Verteporfin (6 mg/m^2) was administered as a standard 10-min infusion, and the PDT laser was applied with a reduced fluence (25 J/cm^2, 300 mW/cm^2) 5 min after the infusion of the drug for 83 s. This was followed by a single intravitreal injection of ranibizumab 0.3 mg/0.05 ml and dexamethasone 200 μg in 0.05 ml that were combined in the same syringe (total volume = 0.1 ml).

The intravitreal injection was administered using a standard sterile technique. Measurement of intra-ocular pressure was performed prior to and 30 min after the injection. A postinjection course of ofloxacin eyedrops 3 times daily for 4 days was prescribed.

Subsequent Treatment Regimen

All subsequent intravitreal injections of ranibizumab only were administered if they fulfilled the retreatment criteria. Oral minocycline 100 mg daily was started the following day and continued for 3 months. Patients were instructed to return all used and unused drug packages to ascertain compliance.

Retreatment Criteria

Re-injection with ranibizumab was indicated if any of the following were present at the monthly follow-up visits:

(1) a loss of >5 letters of refracted logMAR BCVA recorded on the modified ETDRS chart when compared to the previous month;
(2) an increase in central retinal thickness (CRT) of >100 μm measured on the fast macular thickness map when compared to the previous month;
(3) fresh macular haemorrhage on dilated fundus biomicroscopy.

Outcome Measures

The primary efficacy outcome measures were (1) the mean change in BCVA from baseline to study completion, (2) the proportion of patients with stable vision (a loss or gain of less than 15 letters) and (3) the mean ranibizumab retreatment rate per study eye over the follow-up period.

The secondary outcome measures were (1) the proportion of study eyes which gained ≥5 letters of BCVA, (2) the proportion of study eyes which lost ≥15 letters of BCVA, (3) mean final BCVA and (4) the mean change in the CRT on OCT.

The safety profile was assessed by visual acuity, tonometry, ophthalmic examinations, adverse events and vital signs.

Statistical Analysis

The proof-of-concept safety hypothesis used in other combination studies to measure possible safety implications and the relationship between the concomitant administration of two drugs was used in this study (FOCUS and PROTECT) [26–28]. Enrolment of 19 patients was needed to allow a 95% chance of observing at least 1 adverse event with a true rate of occurrence of ≥4% within the treatment period, based on the 3% rate of moderate visual loss reported in the PROTECT study, the study that assessed safety of same-day standard PDT and ranibizumab and the study that assessed outcomes of combined bevazicumab and dexamethasone with PDT that showed that 3.8% lost 3 or more lines [29]. These safety assessments included the incidence of severe vision loss (number of patients losing 30 letters of BCVA from baseline) and the incidence of moderate vision loss (number of patients losing 15 letters of BCVA from baseline).

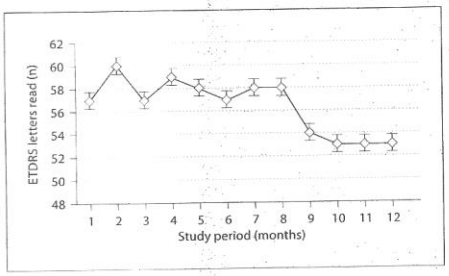

Fig. 1. Mean final visual outcome at 12 months. p = 0.06.

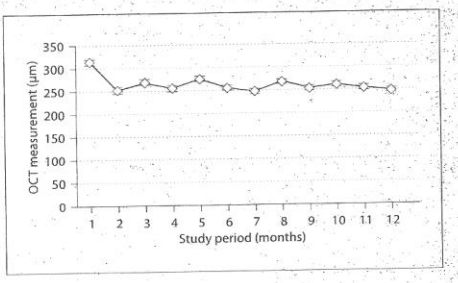

Fig. 2. Mean changes in CRT during the study period. p = 0.00189.

Table 1. The baseline characteristics of the study cohort

Baseline characteristics	Number of eyes (total = 18)
Mean age, years	79.2 ± 6
Laterality (OD:OS)	11:7
CNV type	
Predominantly classic	3 (15.7%)
Minimally classic	3 (15.7%)
Occult not classic	12 (63.2%)
Retinal angiomatous proliferation	1 (5.3%)

Results

Nineteen eyes of 19 patients were recruited into the study. One patient was withdrawn from the study as a result of epidural haematoma after a retreatment at month 2. Three patients were retreated with 0.5 mg/0.05 ml of commercially available ranibizumab (2 from visit 10 and 1 from visit 8) due to non-availability of the study-specific ranibizumab (0.3 mg/0.05 ml) preparation. The baseline characteristics are described in table 1.

Visual Acuity Outcomes

The mean change in BCVA at the final follow-up was –5.0 (±10.5) ETDRS letters (fig. 1). The final mean BCVA was 52.6 ± 16 ETDRS letters (range 7–76). Eighty-nine percent (16/18 eyes) had stable vision losing fewer than 15 ETDRS letters. None of the study eyes gained more than 15 letters, although 3/15 eyes gained more than 5 ETDRS letter (7-, 11- and 12-letter gain). One eye suffered a moderate visual loss of 24 letters, and another eye had severe visual loss of 30 letters.

OCT Outcomes

The mean reduction in CRT was 66.3 (±75) µm. The mean final CRT on OCT was 248 ± 52 µm. The greatest reduction in CRT measurements occurred 1 month after the combined treatment was administered after which it remained relatively stable (fig. 2).

Retreatment Outcomes

The mean total number of ranibizumab injections given was 3.4 (baseline ranibizumab + ranibizumab retreatments). The average number of months from baseline treatment with combined reduced-fluence verteporfin PDT, ranibizumab, dexamethasone and minocycline to retreatment with ranibizumab monotherapy was 2.6 months (range 1–6). Two eyes required 5 ranibizumab retreatments, and the mean changes in visual acuity in these eyes were –12 and –6 ETDRS letters, respectively. Three eyes required only 1 additional ranibizumab retreatment, while the rest of the cohort needed 2–3 injections.

Safety Assessment

One eye suffered a moderate visual loss of 24 letters. This 73-year-old patient had a predominantly classic lesion with a pretreatment baseline visual acuity of 31 letters, CRT of 319 µm and lesion size of 9.08 mm^2. In the first month following baseline combination treatment, the vision improved to 37 letters and CRT was reduced to 182 µm. Two additional ranibizumab retreatments were given at follow-up months 3 and 5. However, the visual

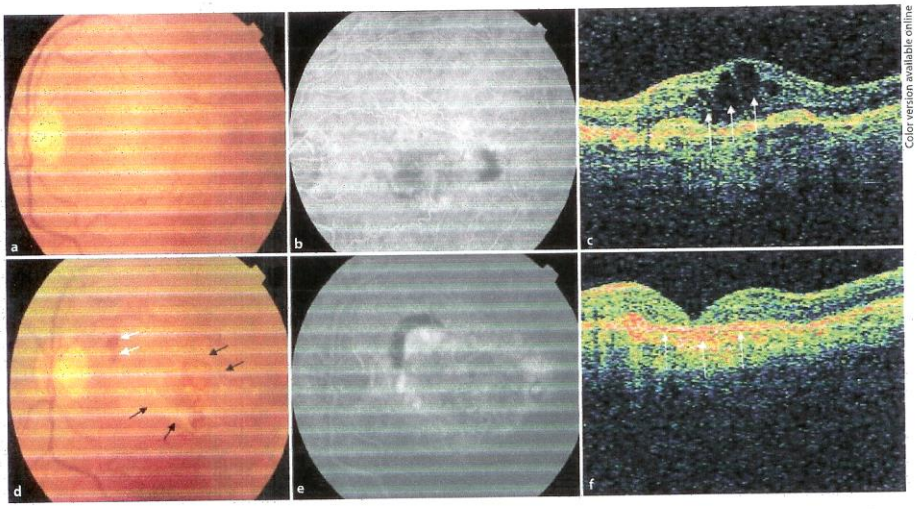

Fig. 3. Fundus colour photograph, fluorescein angiogram and OCT of 73-year-old patient who lost 24 letters at the end of 12 months (**a–f**). **a** Colour photograph at baseline showing minimal pigmentary changes and retinal thickening. **b** Mid-frame fluorescein angiogram at baseline showing areas of leakage. **c** Baseline OCT vertical section through the lesion showing intraretinal fluid (white arrows). **d** Colour photograph showing central areas of fibrosis (black arrows) and fresh eccentric retinal haemorrhage (white arrows). **e** Mid-frame fluorescein angiogram showing new areas of leakage and masking from retinal haemorrhage. **f** OCT at month 12 showing subretinal thickening suggestive of fibrosis (white arrows).

acuity dropped by 16 letters compared to the previous month and continued to progressively deteriorate. The final visual acuity was 7 letters and CRT was 267 μm. The visual loss was attributed to central subretinal fibrosis, and the final size of the fibrotic lesion was 14.502 mm^2 (fig. 3).

Severe visual loss of 30 letters was reported in 1 eye. This 71-year-old patient had an occult lesion with a baseline visual acuity of 62 letters, CRT of 279 μm and lesion size of 9.266 mm^2. Baseline combination therapy and 1 ranibizumab retreatment at follow-up month 3 stabilised the vision for 5 months. Despite 2 further ranibizumab injections, the vision progressively deteriorated from month 6 to a final visual acuity of 32 letters. The final size of the fibrotic lesion was 16.037 mm^2.

None of the patients had any inflammatory response, raised intra-ocular pressure or cataract extraction during the study period.

There was 1 case of mild gastrointestinal disturbance secondary to oral minocycline, but the patient was able to complete the 3-month course. There was 1 study-related death. This 91-year-old patient had received both the combination treatment regime at baseline and a retreatment with ranibizumab at month 2. She then died of an epidural haematoma a week following the treatment.

Discussion

This pilot study was powered to determine the adverse events when a quadruple therapy of reduced-fluence PDT, intravitreal ranibizumab and dexamethasone and oral minocycline are used as a treatment option for neovascular AMD. The efficacy of individual components of the combination therapy is not evaluated in this study. Although it is a negative study, there are few useful mes-

sages that are useful for clinical practice and for future trial designs.

Firstly, the study showed that the quadruple therapy can be delivered safely. Reduced-fluence PDT with 25 J/cm^2 reduces the choroidal hypoperfusion, inflammation, vascular leakage and VEGF upregulation that is associated with standard-fluence PDT [30–32]. It was used only once during the course of treatment in combination with ranibizumab and dexamethasone. Dexamethasone provided additional anti-inflammatory, antifibrotic, antiproliferative and anti-VEGF effects. It is more rapidly cleared from the vitreous than triamcinolone with a lower risk of raised intra-ocular pressure and cataract formation [33, 34]. Oral minocycline, a semisynthetic tetracycline derivative, was used for its immunomodulatory, anti-angiogenic, antimetalloproteinase, anti-oxidant and anti-apoptotic effects [35]. A reduced dose of ranibizumab (0.3 mg in 0.05 ml) was used, given the combined angio-occlusive effect of PDT and the anti-angiogenic effects of dexamethasone and minocycline. No angiographic evidence of choroidal non-perfusion was noted at 6 months in any of the patients in this case series, unlike other combination studies [36]. This may be because choroidal perfusion defects are noted within a week of combination treatment while our study protocol was monthly visits.

Two of the 18 patients (11%) lost more than 3 lines of vision due to subretinal fibrosis. In contrast, the PROTECT study showed that only 1/32 lost more than 15 letters at 9 months. The difference between the two studies was that intravitreal ranibizumab was given monthly for 4 months in the induction phase and PDT was administered at 12 weekly intervals if angiographic leakage was present at 3, 6 and 9 months in the PROTECT study compared to an 'as needed' strategy for re-injection with ranibizumab and no further PDT in this study.

Both patients in this study lost the vision due to subretinal fibrosis, and the increase in fibrosis was noted at the 5th and 8th months, respectively. The second injection of intravitreal ranibizumab was only given at 3 months in both cases, and the change of vision at that point was 0 and +2 and re-injection of ranibizumab was given for recurrence of fluid. This suggests that in combination treatment, retreatment with intravitreal ranibizumab based on recurrent fluid is an insufficient retreatment criterion. This trial was designed before the OCT-guided dosing schedules were published. Clearly, this study shows that it is insufficient to treat recurrent fluid only.

This study also did not show the initial steep gradient of gain in vision noted in the PROTECT study and the ranibizumab monotherapy trials (MARINA and ANCHOR), suggesting that the quadruple therapy at baseline (or only 1 intravitreal ranibizumab injection) is insufficient to improve vision. Repeated injections of ranibizumab in the induction phase are required to achieve an improvement in vision.

The study results correlate well with the observation that reduced levels of aqueous VEGF and pigment epithelium-derived factor are associated with ranibizumab therapy regardless of combined therapy with PDT. The reduction levels of VEGF and pigment epithelium-derived factor also correlated with anatomical improvements in the macula, suggesting that ranibizumab is the main driver of outcomes [37].

Our study results mirror the outcomes of other multicentre trials on combination treatment of ranibizumab and PDT such as the FOCUS study [28].

The MONT BLANC study that evaluated the superiority of standard PDT with ranibizumab compared to ranibizumab monotherapy also showed that an induction of 3 injections of intravitreal ranibizumab is required for an improvement in vision (+7.1 ETDRS letters). The study also showed that a PRN schedule in the maintenance phase of either ranibizumab monotherapy or the combination of standard PDT with ranibizumab booster is insufficient to sustain the improvement of vision [38].

This study reiterates that aggressive retreatment strategies have to be initiated to improve and sustain the effect [39]. Although ranibizumab monotherapy is a burden to patient, treating physicians and health care systems, it is the only treatment that can sustain an improved vision obtained at induction if strict re-treatment criteria are implemented.

Acknowledgement

Investigator-initiated research was funded by Novartis UK.

Disclosure Statement

S. Sivaprasad has received research funds and travel grants, and is a consultant of Novartis, Allergan and Pfizer. N.V. Chong has received travel grants and research funds, and is a consultant of Novartis, Allergan, Pfizer, Merck, Alcon and Bayer.

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Appendix H

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